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<b>(54) Title:</b> MULTIPARAMETER FACS ASSAYS TO DETECT ALTERATIONS IN CELLULAR PARAMETERS AND TO SCREEN SMALL MOLECULE LIBRARIES  <b>(57) Abstract</b>  The invention relates to novel methods of detecting alterations in cell cycle regulation in a cell or a cell population and screening for agents capable of modulating cell cycle regulation through the use of multiparameter assays and a fluorescence-activated cell sorter (FACS) machine.		

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MULTIPARAMETER FACS ASSAYS TO DETECT  
ALTERATIONS IN CELLULAR PARAMETERS AND TO SCREEN SMALL MOLECULE LIBRARIES

FIELD OF THE INVENTION

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The invention relates to novel methods of detecting alterations in cellular parameters, and particularly for screening libraries of small molecules such as combinatorial chemical libraries of organic molecules, including peptides and other chemical libraries, for binding to target molecules, using fluorescence-activated cell sorting (FACS) machines.

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BACKGROUND OF THE INVENTION

The field of drug discovery and screening of drug candidates to identify lead compounds is rapidly expanding. Traditional approaches to identify and characterize new and useful drug candidates include the isolation of natural products or synthetic preparation, followed by testing against either known or unknown targets. See for example WO 94/24314, Gallop et al., J. Med. Chem. 37(9):1233 (1994); Gallop et al., J. Med. Chem. 37(10):1385 (1994); Ellman, Acc. Chem. Res. 29:132 (1996); Gordon et al., E. J. Med. Chem. 30:388s (1994); Gordon et al., Acc. Chem. Res. 29:144 (1996); WO 95/12608, all of which are incorporated by reference.

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The screening of these libraries is done in a variety of ways. One approach involves attachment to beads and visualization with dyes; see Neslter et al., Bioorg. Med. Chem. Lett. 6(12):1327 (1996). Another approach has utilized beads and fluorescence activated cell sorting (FACS); see Needles et al., PNAS USA 90:10700 (1993), and Vetter et al., Bioconjugate Chem. 6:319 (1995).

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Fluorescence activated cell sorting (FACS), also called flow cytometry, is used to sort individual cells on the basis of optical properties, including fluorescence. It is generally fast, and can result in screening large populations of cells in a relatively short period of time.

There are a number of instances where rapid and inexpensive screens such as FACS screens would be of particular interest. One such area is in cell cycle assays. Cells cycle through various stages of growth, starting with the M phase, where mitosis and cytoplasmic division (cytokinesis) occurs. The M phase is followed by the G1 phase, in which the cells resume a high rate of biosynthesis and growth. The S phase begins with DNA synthesis, and ends when the DNA content of the nucleus has doubled. The cell then enters G2 phase, which ends when mitosis starts, signaled by the appearance of condensed chromosomes. Terminally differentiated cells are arrested in the G1 phase, and no longer undergo cell division.

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The hallmark of a malignant cell is uncontrolled proliferation. This phenotype is acquired through the accumulation of gene mutations, the majority of which promote passage through the cell cycle. Cancer cells ignore growth regulatory signals and remain committed to cell division. Classic oncogenes, such as *ras*, lead to inappropriate transition from G1 to S phase of the cell cycle, mimicking proliferative extracellular signals. Cell cycle checkpoint controls ensure faithful replication and segregation of the genome. The loss of cell cycle checkpoint control results in genomic instability, greatly accelerating the accumulation of mutations which drive malignant transformation. Hence, checkpoint regulators, such as p53 and ATM (ataxia telangiectasia mutated), also function as tumor suppressors. Thus, modulating cell cycle checkpoint pathways with therapeutic agents could exploit the differences between normal and tumor cells, both improving the selectivity of radio- and chemotherapy, and leading to novel cancer treatments.

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Accordingly, it is an object of the invention to provide compositions and methods useful in screening for modulators of cell cycle checkpoint regulation.

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Another area for which rapid screening methods would find particular use is in the area of assays of exocytosis. Exocytosis is the fusion of secretory vesicles with the cellular plasma membrane, and has two main functions. One is the discharge of the vesicle contents into the extracellular space, and the second is the incorporation of new proteins and lipids into the plasma membrane itself.

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Exocytosis can be divided into two classes: constitutive and regulated. All eukaryotic cells exhibit constitutive exocytosis, which is marked by the continuous fusion of the secretory vesicles after formation. Regulated exocytosis is restricted to certain cells, including exocrine, endocrine and neuronal cells. Regulated exocytosis results in the accumulation of the secretory vesicles that fuse with the plasma membrane only upon receipt of an appropriate signal, usually (but not always) an increase in the cytosolic free  $Ca^{2+}$  concentration.

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- Regulated exocytosis is crucial to many specialized cells, and often a particular cell can release multiple mediators from the same exocytic granules which work in concert to produce a coordinated physiological response in the target cells. These regulated exocytic cells include neurons (neurotransmitter release), adrenal chromaffin cells (adrenaline secretion), pancreatic acinar cells (digestive enzyme secretion), pancreatic  $\beta$ -cells (insulin secretion), mast cells (histamine secretion), mammary cells (milk protein secretion), sperm (enzyme secretion), egg cells (creation of fertilization envelope) and adipocytes (insertion of glucose transporters into the plasma membrane). In addition, current theory suggests that the basic mechanisms of vesicle docking and fusion is conserved from yeast to the mammalian brain.
- In addition, disorders involving exocytosis are known. For example, inflammatory mediator release from mast cells leads to a variety of disorders, including asthma. In the United States alone, over 50 million people suffer from asthma, rhinitis, or some other form of allergy. Therapy for allergy remains limited to blocking the mediators released by mast cells (anti-histamines), non-specific anti-inflammatory agents such as steroids and mast cell stabilizers which are only marginally effective at limiting the symptomatology of allergy. Similarly, Chediak-Higashi Syndrome (CHS) is a rare autosomal recessive disease in which neutrophils, monocytes and lymphocytes and most cells contain giant cytoplasmic granules. Similar disorders have been described in mice, mink, cattle, cats and killer whales, with the murine homolog of CHS (designated *beige* or *bg*) being the best characterized. See Perou et al., J. Biol. Chem. 272(47):29790 (1997) and Barbosa et al., Nature 382:262 (1996), both of which are hereby incorporated by reference.

Furthermore, it is widely believed that a wide array of psychiatric disorders are the result of an imbalance between neurotransmitter exocytosis and mediator reuptake.

- A large number of pharmaceuticals have been designed to specifically interfere with the exocytic mediators primarily through blockade of their receptors. However, this approach is limited by the fact that a single receptor blocker cannot overcome the effects of many diverse mediators.
- Accordingly, it is an object of the present invention to provide methods for screening for alterations in exocytosis, particularly for screening for agents capable of mediating such exocytosis. It is also an object to provide such screening methods wherein assay background is reduced and specificity is increased.

## SUMMARY OF THE INVENTION

In accordance with the objects outlined above, the present invention provides methods for screening

bioactive agents for the ability to alter or modulate alterations in cellular phenotypes. The methods generally comprise combining at least one candidate bioactive agent and a population of cells, sorting the cells in a FACS machine by separating the cells on the basis of at least three, four or five cellular parameters. The candidate agents can be part of a molecular library comprising fusion nucleic acids  
5 encoding the candidate bioactive agents.

In a further aspect, the present invention provides methods for screening for alterations in exocytosis of a population of cells or in single cells under different conditions or combined with different bioactive agents. The methods comprise sorting the cells in a FACS machine by assaying for alterations in at  
10 least three of the properties selected from the group consisting of light scattering, fluorescent dye uptake, fluorescent dye release, annexin granule binding, surface granule enzyme activity, and the quantity of granule specific proteins.

Also provided herein is a method for screening for a bioactive agent capable of modulating exocytosis  
15 in a cell. This method comprises combining a candidate bioactive agent and a population of cells and subjecting said cells to conditions that normally cause exocytosis. The cells are sorted in a FACS machine by assaying for alterations in at least three of the properties selected from the group consisting of light scattering, fluorescent dye uptake, fluorescent dye release, annexin granule binding, surface granule enzyme activity, and the quantity of granule specific proteins. Alterations in at least  
20 one of said properties as compared to cells that were not exposed to the candidate bioactive agent indicates that said agent modulates exocytosis.

In a preferred embodiment of the method for screening for a bioactive agent, the properties selected include at least one property selected from the group consisting of fluorescent dye release, annexin  
25 granule binding, surface granule enzyme activity, and the quantity of granule specific proteins.

When fluorescent dye uptake is detected, the dye is preferably a styryl dye. In the case that fluorescent dye release is detected, the dye can be a low pH concentration dye or a styryl dye.

30 In a preferred embodiment, the surface granule enzyme activity is detected by an in situ enzymology assay or by a population based enzyme assay. The enzyme substrate can be any detectable substrate. Preferably, the enzyme substrate is coupled to a FRET construct. FRET constructs include two fluorescent proteins divided by a protease site. In this case, the protease site is specific for a granule protease.

35 In a preferred embodiment, granule specific proteins are detected. The granule specific proteins can be any detectable protein. In one embodiment, the granule specific proteins are fusion proteins

comprising a granule specific protein and a detectable molecule which can be a FRET construct.

In another preferred embodiment, a method for screening for a bioactive agent capable of modulating exocytosis in a cell is provided wherein said method comprises combining at least one candidate  
5 bioactive agent and a population of cells each containing a fusion nucleic acid comprising a nucleic acid encoding a granule-specific protein and a label. The cells are subjected to conditions that normally cause exocytosis and the alterations in the quantity of the label is detected. Alterations in the quantity of the label indicates that the agent modulates exocytosis. Preferably, the label is an epitope tag or a fluorescent molecule. In a preferred embodiment, the fluorescent molecule is a FRET  
10 construct.

In an additional aspect, the invention provides methods of screening for exocytosis modulators comprising combining candidate bioactive agents, cells comprising nucleic acids encoding a detectable granule-specific protein, and an agent for detecting this protein. The cells are subjected to  
15 conditions that normally cause exocytosis, and the presence or absence of the protein is determined.

In another preferred embodiment, a method for screening for a bioactive agent capable of modulating exocytosis in a cell is provided which comprises combining at least one candidate bioactive agent and a population of cells. The cells are subjected to conditions that normally cause exocytosis and a  
20 fluorescent annexin is added. Alterations in the amount of the fluorescent annexin on the surface of the cells is evaluated.

In another preferred embodiment, a method for screening for a bioactive agent capable of modulating exocytosis in a cell is provided which comprises providing a population of cells wherein the cells have  
25 taken in a low pH concentration dye. The low pH concentration dye loaded cells are combined with at least one candidate bioactive agent and subjected to conditions that normally cause exocytosis. The release of the low pH concentration dye is detected. Alterations in the amount of released dye indicate that the agent modulates exocytosis.

In another preferred embodiment, a method for screening for a bioactive agent capable of modulating exocytosis in a cell is provided which comprises combining at least one candidate bioactive agent and a population of cells. The cells are subjected to conditions that normally cause exocytosis and a  
30 fluorescent substrate specific to a granule enzyme is added. The fluorescent substrate specific to a granule enzyme is detected, wherein alterations in the amount of the fluorescent substrate indicative  
35 that the agent modulates exocytosis. In a preferred embodiment, the substrate comprises a FRET construct.

In an additional aspect, the present invention provides methods and compositions for screening for bioactive agents capable of modulating cell cycle regulation in a cell. The method comprises combining a library of candidate bioactive agents and a population of cells, sorting the cells in a FACS machine by separating the cells on the basis of at least a cell viability assay, a proliferation assay, and  
5 a cell phase assay.

In a further aspect, the methods comprise expressing a library of fusion nucleic acids in a library of cells. The fusion nucleic acids comprise a nucleic acid encoding a candidate bioactive agent and a detectable moiety. The cells are sorted in a FACS machine by separating the cells; when the cellular  
10 phenotype is cell cycle, the cells are sorted on the basis of at least a cell viability assay, an expression assay, a proliferation assay, and a cell phase assay.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15 Figures 1A, 1B and 1C schematically depict three retroviral constructions of Example 1. Figure 1A includes the CRU5-GFP-p21 construction, comprising a CRU5 promoter, the  $\psi$ -retroviral packaging signal, the coding region for GFP, fused to the coding region of p21, followed by an LTR. Figure 1B depicts the CRU5-GFP-p21C construction, which includes the C-terminal 24 amino acids of p21. Figure 1C depicts the CRU5-GFP-pUCmut construct, which is a mutant version of CRU5-p21C with 3  
20 alanine substitutions.

Figures 2A, 2B, 2C and 2D depict the results of the experiments of Example 1. Figure 2A depicts a viability assay utilizing forward and side scatter. Cells exhibiting a characteristic ratio are collected. Figure 2B shows the fluorescence of the GFP of the vectors. Figure 2C depicts the use of PKH26, an  
25 inclusion dye, in a proliferation assay; the cells containing p21, a protein known to arrest cells, remain brightly fluorescent, while the control cells continue to proliferate, thus diluting the dye and losing fluorescence. Figure 2D depicts the use of Hoechst 33342 in a cell phase assay.

Figure 3 depicts the effect of AraC treatment on Jurkat cells infected with p21, an agent that arrests  
30 cells in the G1 phase. AraC is a nucleotide analog that is toxic to dividing cells. Thus, those cells that are cell cycle arrested survive. The lower line depicts Jurkat cells without the p21 insert, and the upper line depicts Jurkat cells with the p21 insert.

Figures 4A and 4B depict bar graphs showing the results of a population based exocytic enzyme  
35 activity assay for exocytosis. Figure 4A shows glucuronidase or hexosaminidase activity in the supernatant of cells combined with DMSO (-) or ionomycin (+). Figure 4B shows hexosaminidase activity in the supernatant of cells sensitized with varying amounts of IgE anti-DNP and stimulated with



increasing amounts of the antigen BSA-DNP.

Figures 5A-5F show exocytic light scatter changes observed on the flow cytometer, side scatter vs. forward scatter, plotted as bivariate histograms for RBL-2H3 cells (Figures 5A and 5D) and MC-9 cells (Figures 5B, 5C, 5E and 5F). After stimulation with an ionophore, the cells were observed at 0 minutes (Figures 5A and 5C), 5 minutes (Figure 5E), 10 minutes (Figure 5D), and 30 minutes (Figures 5B and 5F).

Figures 6A-6E show graphs of the results of a styryl dye assay to detect exocytosis by FACS. Cells were combined with (left peaks) DMSO or (right peaks) ionomycin in the presence of either FM 4-64 (Figures 6A and 6B) or FM 1-43 (Figures 6C, 6D and 6E). Figures 6A and 6C show cells detected in fluorescence channel 1. Figures 6B and 6D show cells detected in fluorescence channel 3. Figure 6E shows the mean channel shift detected in the flow cytometer in fluorescence channel 1 plotted as a bar graph wherein cells were preincubated with varying doses of the PI-3 kinase inhibitor wortmannin prior to administration of an ionophore (bars 1-4) or DMSO (bar 5) in the presence of FM 1-43.

Figures 7A-7D show graphs depicting the results of an annexin-V detection assay of exocytosis by FACS. Cells were combined with either DMSO (Figures 7A and 7B) or ionomycin (Figures 7C and 7D) and then stained with both propidium iodide (Figures 7A and 7C) and annexin-V-FITC (Figures 7B and 7D).

Figures 8A-8C show graphs indicating the results of an in situ enzymology assay of exocytosing cells visualized by FACS. Cells were combined with DMSO (Figure 8A) or an ionophore (Figures 8B and 8C) and then stained for in situ glucuronidase activity. Figure 8C shows the pH profile of the cell surface enzymatic activity wherein the bar graphs represent the percentage of maximal signal, as measured by mean channel shift in the flow cytometer, observed.

Figure 9 is a histogram of fluorescence intensity detected in channel 1 showing cells loaded with LYSOTRACKER GREEN™, combined with either DMSO (left) or ionomycin (right) and viewed in the flow cytometer.

Figures 10A-10H show the results of a multiparameter analysis including detection of LYSOTRACKER GREEN™, annexin-V-APC and forward and side scatter. Figures 10A-10D and 10E-10H each show cells treated with increasing doses of ionomycin and observed in the flow cytometer with four parameters simultaneously. The cells were loaded with low pH concentration dye, stimulated and stained with annexin-V-APC. Figures 10A-10D show bivariate histograms of side vs. forward light scatter and Figures 10E-10H show bivariate histograms of annexin-V-APC vs. low pH concentration

dye signals.

Figure 11 shows a graph of cells stimulated in the presence of FM 1-43 and annexin-V-APC stained. At various timepoints after ionomycin stimulation the cells were analyzed by flow cytometry and the supernatant for enzymatic activity (cell supernatant). The parameters forward scatter, FM-143, annexin-V-APC, and hexosaminidase are plotted on the graph relative to the maximal response for each parameter. For calcium signaling, a separate tube of cells was loaded with Fluo-3 and underwent the identical procedure.

## 10 DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the detection of alterations in cellular phenotypes, such as cell cycle regulation, exocytosis, small molecule toxicity, cell surface receptor expression, enzyme expression, etc. by evaluating or assaying a variety of cellular parameters, generally through the use of a fluorescence-activated cell sorter (FACS) machine. There are a number of parameters that can be measured to allow detection of alterations in a variety of cellular phenotypes as is more fully outlined below. By assaying a plurality of these parameters either sequentially or preferably simultaneously, rapid and accurate screening may be done.

20 In a preferred embodiment, the methods outlined herein are used to screen for modulators of cellular phenotypes. Cellular phenotypes that may be assayed include, but are not limited to, cellular apoptosis, including cell cycle regulation, exocytosis, toxicity to small molecules, the expression of any number of moieties including receptors (particularly cell surface receptors), adhesion molecules, cytokine secretion, protein-protein interactions, etc.

25 In a preferred embodiment, the methods are used to evaluate cell cycle regulation. In this embodiment, preferred cellular parameters or assays are cell viability assays, assays to determine whether cells are arrested at a particular cell cycle stage ("cell proliferation assays"), and assays to determine at which cell stage the cells have arrested ("cell phase assays"). By assaying or measuring one or more of these parameters, it is possible to detect not only alterations in cell cycle regulation, but alterations of different steps of the cell cycle regulation pathway. This may be done to evaluate native cells, for example to quantify the aggressiveness of a tumor cell type, or to evaluate the effect of candidate drug agents that are being tested for their effect on cell cycle regulation. In this manner, rapid, accurate screening of candidate agents may be performed to identify agents that modulate cell cycle regulation.

Thus, the present methods are useful to elucidate bioactive agents that can cause a population of cells

to either move out of one growth phase and into another, or arrest in a growth phase. In some embodiments, the cells are arrested in a particular growth phase, and it is desirable to either get them out of that phase or into a new phase. Alternatively, it may be desirable to force a cell to arrest in a phase, for example G1, rather than continue to move through the cell cycle. Similarly, it may be  
5 desirable in some circumstances to accelerate a non-arrested but slowly moving population of cells into either the next phase or just through the cell cycle, or to delay the onset of the next phase. For example, it may be possible to alter the activities of certain enzymes, for example kinases, phosphatases, proteases or ubiquitination enzymes, that contribute to initiating cell phase changes.

10 In a preferred embodiment, the methods outlined herein are done on cells that are not arrested in the G1 phase; that is, they are rapidly or uncontrollably growing and replicating, such as tumor cells. In this manner, candidate agents are evaluated to find agents that can alter the cell cycle regulation, i.e. cause the cells to arrest at cell cycle checkpoints, such as in G1 (although arresting in other phases such as S, G2 or M are also desirable). Alternatively, candidate agents are evaluated to find agents  
15 that can cause proliferation of a population of cells, i.e. that allow cells that are generally arrested in G1 to start proliferating again; for example, peripheral blood cells, terminally differentiated cells, stem cells in culture, etc.

Accordingly, in a preferred embodiment, the invention provides methods for screening for alterations in  
20 cell cycle regulation of a population of cells. "Alteration" and "modulation" (used herein interchangeably), as used herein can include both increases and decreases in the parameter or phenotype being measured. By "alteration" or "modulation" in the context of cell cycle regulation, is generally meant one of two things. In a preferred embodiment, the alteration results in a change in the cell cycle of a cell, i.e. a proliferating cell arrests in any one of the phases, or an arrested cell moves  
25 out of its arrested phase and starts the cell cycle, as compared to another cell or in the same cell under different conditions. Alternatively, the progress of a cell through any particular phase may be altered; that is, there may be an acceleration or delay in the length of time it takes for the cells to move thorough a particular growth phase. For example, the cell may be normally undergo a G1 phase of several hours; the addition of an agent may prolong the G1 phase.

30 The measurements can be determined wherein all of the conditions are the same for each measurement, or under various conditions, with or without bioactive agents, or at different stages of the cell cycle process. For example, a measurement of cell cycle regulation can be determined in a cell population wherein a candidate bioactive agent is present and wherein the candidate bioactive  
35 agent is absent. In another example, the measurements of cell cycle regulation are determined wherein the condition or environment of the populations of cells differ from one another. For example, the cells may be evaluated in the presence or absence of physiological signals, for example

hormones, antibodies, peptides, antigens, cytokines, growth factors, action potentials, pharmacological agents (i.e. chemotherapeutics, etc.), or other cells (i.e. cell-cell contacts). In another example, the measurements of cell cycle regulation are determined at different stages of the cell cycle process. In yet another example, the measurements of cell cycle regulation are taken wherein the  
5 conditions are the same, and the alterations are between one cell or cell population and another cell or cell population.

By a "population of cells" or "library of cells" or "plurality of cells" herein is meant at least two cells, with at least about  $10^3$  being preferred, at least about  $10^6$  being particularly preferred, and at least about  
10  $10^8$  to  $10^9$  being especially preferred. The population or sample can contain a mixture of different cell types from either primary or secondary cultures although samples containing only a single cell type are preferred, for example, the sample can be from a cell line, particularly tumor cell lines (particularly when, as outlined below. The cells may be in any cell phase, either synchronously or not, including M, G1, S, and G2. In a preferred embodiment, cells that are replicating or proliferating are used; this  
15 may allow the use of retroviral vectors for the introduction of candidate bioactive agents. Alternatively, non-replicating cells may be used, and other vectors (such as adenovirus and lentivirus vectors) can be used. In addition, although not required, the cells are compatible with dyes and antibodies.

Preferred cell types for use in the invention will vary with the cellular phenotype to be modulated.  
20 Suitable cells include, but are not limited to, mammalian cells, including animal (rodents, including mice, rats, hamsters and gerbils), primates, and human cells, particularly including tumor cells of all types, including breast, skin, lung, cervix, colonrectal, leukemia, brain, etc. As outlined below, additional cell types may be used for screening for exocytosis.

25 In a preferred embodiment, the cell cycle regulation methods comprise sorting the cells in a FACS machine by assaying several different cell parameters, including, but not limited to, cell viability, cell proliferation, and cell phase.

In a preferred embodiment, cell viability is assayed, to ensure that a lack of cellular change is due to  
30 experimental conditions (i.e. the introduction of a candidate bioactive agent) not cell death. There are a variety of suitable cell viability assays which can be used, including, but not limited to, light scattering, viability dye staining, and exclusion dye staining.

In a preferred embodiment, a light scattering assay is used as the viability assay, as is well known in  
35 the art. When viewed in the FACS, cells have particular characteristics as measured by their forward and 90 degree (side) light scatter properties. These scatter properties represent the size, shape and granule content of the cells. These properties account for two parameters to be measured as a

readout for the viability. Briefly, the DNA of dying or dead cells generally condenses, which alters the 90° scatter; similarly, membrane blebbing can alter the forward scatter. Alterations in the intensity of light scattering, or the cell-refractive index indicate alterations in viability.

- 5 Thus, in general, for light scattering assays, a live cell population of a particular cell type is evaluated to determine its forward and side scattering properties. This sets a standard for scattering that can subsequently be used.

- 10 In a preferred embodiment, the viability assay utilizes a viability dye. There are a number of known viability dyes that stain dead or dying cells, but do not stain growing cells. For example, annexin V is a member of a protein family which displays specific binding to phospholipid (phosphatidylserine) in a divalent ion dependent manner. This protein has been widely used for the measurement of apoptosis (programmed cell death) as cell surface exposure of phosphatidylserine is a hallmark early signal of this process. Suitable viability dyes include, but are not limited to, annexin, ethidium homodimer-1,  
15 DEAD Red, propidium iodide, SYTOX Green, etc., and others known in the art; see the Molecular Probes Handbook of Fluorescent Probes and Research Chemicals, Haugland, Sixth Edition, hereby incorporated by reference; see Apoptosis Assay on page 285 in particular, and Chapter 16.

- 20 Protocols for viability dye staining for cell viability are known, see Molecular Probes catalog, supra. In this embodiment, the viability dye such as annexin is labeled, either directly or indirectly, and combined with a cell population. Annexin is commercially available, i.e., from PharMingen, San Diego, California, or Caltag Laboratories, Millbrae, California. Preferably, the viability dye is provided in a solution wherein the dye is in a concentration of about 100 ng/ml to about 500 ng/ml, more preferably, about 500 ng/ml to about 1 µg/ml, and most preferably, from about 1 µg/ml to about 5 µg/ml. In a  
25 preferred embodiment, the viability dye is directly labeled; for example, annexin may be labeled with a fluorochrome such as fluorescein isothiocyanate (FITC), Alexa dyes, TRITC, AMCA, APC, tri-color, Cy-5, and others known in the art or commercially available. In an alternate preferred embodiment, the viability dye is labeled with a first label, such as a hapten such as biotin, and a secondary fluorescent label is used, such as fluorescent streptavidin. Other first and second labeling pairs can be used as  
30 will be appreciated by those in the art.

Once added, the viability dye is allowed to incubate with the cells for a period of time, and washed, if necessary. The cells are then sorted as outlined below to remove the non-viable cells.

- 35 In a preferred embodiment, exclusion dye staining is used as the viability assay. Exclusion dyes are those which are excluded from living cells, i.e. they are not taken up passively (they do not permeate the cell membrane of a live cell). However, due to the permeability of dead or dying cells, they are

taken up by dead cells. Generally, but not always, the exclusion dyes bind to DNA, for example via intercalation. Preferably, the exclusion dye does not fluoresce, or fluoresces poorly, in the absence of DNA; this eliminates the need for a wash step. Alternatively, exclusion dyes that require the use of a secondary label may also be used. Preferred exclusion dyes include, but are not limited to, ethidium bromide; ethidium homodimer-1; propidium iodine; SYTOX green nucleic acid stain; Calcein AM, BCECF AM; fluorescein diacetate; TOTO® and TO-PRO™ (from Molecular Probes; supra, see chapter 16) and others known in the art.

10 Protocols for exclusion dye staining for cell viability are known, see the Molecular Probes catalog, supra. In general, the exclusion dye is added to the cells at a concentration of from about 100 ng/ml to about 500 ng/ml, more preferably, about 500 ng/ml to about 1 µg/ml, and most preferably, from about 0.1 µg/ml to about 5 µg/ml, with about 0.5 µg/ml being particularly preferred. The cells and the exclusion dye are incubated for some period of time, washed, if necessary, and then the cells sorted as outlined below, to remove non-viable cells from the population.

15 In addition, there are other cell viability assays which may be run, including for example enzymatic assays, which can measure extracellular enzymatic activity of either live cells (i.e. secreted proteases, etc.), or dead cells (i.e. the presence of intracellular enzymes in the media; for example, intracellular proteases, mitochondrial enzymes, etc.). See the Molecular Probes Handbook of Fluorescent Probes and Research Chemicals, Haugland, Sixth Edition, hereby incorporated by reference; see chapter 16 in particular.

25 In a preferred embodiment, at least one cell viability assay is run, with at least two different cell viability assays being preferred, when the fluors are compatible. When only 1 viability assay is run, a preferred embodiment utilizes light scattering assays (both forward and side scattering). When two viability assays are run, preferred embodiments utilize light scattering and dye exclusion, with light scattering and viability dye staining also possible, and all three being done in some cases as well. Viability assays thus allow the separation of viable cells from non-viable or dying cells.

30 In addition to a cell viability assay, a preferred embodiment utilizes a cell proliferation assay. By "proliferation assay" herein is meant an assay that allows the determination that a cell population is either proliferating, i.e. replicating, or not replicating.

35 In a preferred embodiment, the proliferation assay is a dye inclusion assay. A dye inclusion assay relies on dilution effects to distinguish between cell phases. Briefly, a dye (generally a fluorescent dye as outlined below) is introduced to cells and taken up by the cells. Once taken up, the dye is trapped in the cell, and does not diffuse out. As the cell population divides, the dye is proportionally diluted. That

is, after the introduction of the inclusion dye, the cells are allowed to incubate for some period of time; cells that lose fluorescence over time are dividing, and the cells that remain fluorescent are arrested in a non-growth phase.

- 5 Generally, the introduction of the inclusion dye may be done in one of two ways. Either the dye cannot passively enter the cells (e.g. it is charged), and the cells must be treated to take up the dye; for example through the use of a electric pulse. Alternatively, the dye can passively enter the cells, but once taken up, it is modified such that it cannot diffuse out of the cells. For example, enzymatic modification of the inclusion dye may render it charged, and thus unable to diffuse out of the cells. For  
10 example, the Molecular Probes CellTracker™ dyes are fluorescent chloromethyl derivatives that freely diffuse into cells, and then glutathione S-transferase-mediated reaction produces membrane impermeant dyes.

- Suitable inclusion dyes include, but are not limited to, the Molecular Probes line of CellTracker™ dyes  
15 , including, but not limited to CellTracker™ Blue, CellTracker™ Yellow-Green, CellTracker™ Green, CellTracker™ Orange, PKH26 (Sigma), and others known in the art; see the Molecular Probes Handbook, supra; chapter 15 in particular.

- In general, inclusion dyes are provided to the cells at a concentration ranging from about  
20 100 ng/ml to about 5 µg/ml, with from about 500 ng/ml to about 1 µg/ml being preferred. A wash step may or may not be used. In a preferred embodiment, a candidate bioactive agent is combined with the cells as described herein. The cells and the inclusion dye are incubated for some period of time, to allow cell division and thus dye dilution. The length of time will depend on the cell cycle time for the particular cells; in general, at least about 2 cell divisions are preferred, with at least about 3 being  
25 particularly preferred and at least about 4 being especially preferred. The cells are then sorted as outlined below, to create populations of cells that are replicating and those that are not. As will be appreciated by those in the art, in some cases, for example when screening for anti-proliferation agents, the bright (i.e. fluorescent) cells are collected; in other embodiments, for example for screening for proliferation agents, the low fluorescence cells are collected. Alterations are determined  
30 by measuring the fluorescence at either different time points or in different cell populations, and comparing the determinations to one another or to standards.

- In a preferred embodiment, the proliferation assay is an antimetabolite assay. In general, antimetabolite assays find the most use when agents that cause cellular arrest in G1 or G2 resting  
35 phase is desired. In an antimetabolite proliferation assay, the use of a toxic antimetabolite that will kill dividing cells will result in survival of only those cells that are not dividing. Suitable antimetabolites include, but are not limited to, standard chemotherapeutic agents such as methotrexate, cisplatin,

taxol, hydroxyurea, nucleotide analogs such as AraC, etc. In addition, antimetabolite assays may include the use of genes that cause cell death upon expression.

5 The concentration at which the antimetabolite is added will depend on the toxicity of the particular antimetabolite, and will be determined as is known in the art. The antimetabolite is added and the cells are generally incubated for some period of time; again, the exact period of time will depend on the characteristics and identity of the antimetabolite as well as the cell cycle time of the particular cell population. Generally, a time sufficient for at least one cell division to occur.

10 In a preferred embodiment, at least one proliferation assay is run, with more than one being preferred. Thus, a proliferation assay results in a population of proliferating cells and a population of arrested cells.

15 In a preferred embodiment, either after or simultaneously with one or more of the proliferation assays outlined above, at least one cell phase assay is done. A "cell phase" assay determines at which cell phase the cells are arrested, M, G1, S, or G2.

20 In a preferred embodiment, the cell phase assay is a DNA binding dye assay. Briefly, a DNA binding dye is introduced to the cells, and taken up passively. Once inside the cell, the DNA binding dye binds to DNA, generally by intercalation, although in some cases, the dyes can be either major or minor groove binding compounds. The amount of dye is thus directly correlated to the amount of DNA in the cell, which varies by cell phase; G2 and M phase cells have twice the DNA content of G1 phase cells, and S phase cells have an intermediate amount, depending on at what point in S phase the cells are. Suitable DNA binding dyes are permeant, and include, but are not limited to, Hoechst 33342 and 25 33258, acridine orange, 7-AAD, LDS 751, DAPI, and SYTO 16, Molecular Probes Handbook, supra; chapters 8 and 16 in particular.

30 In general, the DNA binding dyes are added in concentrations ranging from about 1 µg/ml to about 5 µg/ml. The dyes are added to the cells and allowed to incubate for some period of time; the length of time will depend in part on the dye chosen. In one embodiment, measurements are taken immediately after addition of the dye. The cells are then sorted as outlined below, to create populations of cells that contain different amounts of dye, and thus different amounts of DNA; in this way, cells that are replicating are separated from those that are not. As will be appreciated by those in the art, in some cases, for example when screening for anti-proliferation agents, cells with the least fluorescence (and 35 thus a single copy of the genome) can be separated from those that are replicating and thus contain more than a single genome of DNA. Alterations are determined by measuring the fluorescence at either different time points or in different cell populations, and comparing the determinations to one



another or to standards.

- In a preferred embodiment, the cell phase assay is a cyclin destruction assay. In this embodiment, prior to screening (and generally prior to the introduction of a candidate bioactive agent, as outlined below), a fusion nucleic acid is introduced to the cells. The fusion nucleic acid comprises nucleic acid encoding a cyclin destruction box and a nucleic acid encoding a detectable molecule. "Cyclin destruction boxes" are known in the art and are sequences that cause destruction via the ubiquitination pathway of proteins containing the boxes during particular cell phases. That is, for example, G1 cyclins may be stable during G1 phase but degraded during S phase due to the presence of a G1 cyclin destruction box. Thus, by linking a cyclin destruction box to a detectable molecule, for example green fluorescent protein, the presence or absence of the detectable molecule can serve to identify the cell phase of the cell population. In a preferred embodiment, multiple boxes are used, preferably each with a different fluor, such that detection of the cell phase can occur.
- A number of cyclin destruction boxes are known in the art, for example, cyclin A has a destruction box comprising the sequence RTVLGVIGD; the destruction box of cyclin B1 comprises the sequence RTALGDIGN. See Glotzer et al., Nature 349:132-138 (1991). Other destruction boxes are known as well: YMTVSIIDRFMQDSCVPKKMLQLVGVT (rat cyclin B); KFRLQETMYMTVSIIDRFMQNSCVPKK (mouse cyclin B); RAILIDWLIQVQMKFRLQETMYMTVS (mouse cyclin B1); DRFLQAQLVCRKKLQVVGITALLASK (mouse cyclin B2); and MSVLRGKLQLVGTAAMLL (mouse cyclin A2).

- The nucleic acid encoding the cyclin destruction box is operably linked to nucleic acid encoding a detectable molecule. The fusion proteins are constructed by methods known in the art. For example, the nucleic acids encoding the destruction box is ligated to a nucleic acid encoding a detectable molecule. By "detectable molecule" herein is meant a molecule that allows a cell or compound comprising the detectable molecule to be distinguished from one that does not contain it, i.e., an epitope, sometimes called an antigen TAG, a specific enzyme, or a fluorescent molecule. Preferred fluorescent molecules include but are not limited to green fluorescent protein (GFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), red fluorescent protein (RFP), and enzymes including luciferase and  $\beta$ -galactosidase. When antigen TAGs are used, preferred embodiments utilize cell surface antigens. The epitope is preferably any detectable peptide which is not generally found on the cytoplasmic membrane, although in some instances, if the epitope is one normally found on the cells, increases may be detected, although this is generally not preferred. Similarly, enzymatic detectable molecules may also be used; for example, an enzyme that generates a novel or chromogenic product.

Accordingly, the results of sorting after cell phase assays generally result in at least two populations of

cells that are in different cell phases.

In a preferred embodiment, the methods are used to screen candidate bioactive agents for the ability to modulate cell cycle regulation, including the activation or suppression of cell cycle checkpoint pathways and ameliorating checkpoint defects. The candidate bioactive agent can be added to the cell population exogenously or can be introduced into the cells as described further herein.

The term "candidate bioactive agent" or "exogeneous compound" as used herein describes any molecule, e.g., protein, small organic molecule, carbohydrates (including polysaccharides), polynucleotide, lipids, etc. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration or below the level of detection. In addition, positive controls can be used. For example, in the cell cycling assays, agents known to alter cell cycling may be used. For example, p21 is a molecule known to arrest cells in the G1 cell phase, by binding G1 cyclin-CDK complexes. Similarly, for exocytosis, compounds known to induce exocytosis can be used as is more fully outlined below.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are peptides.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

In a preferred embodiment, the candidate bioactive agents are proteins. By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein means  
5 both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and noreleucine are considered amino acids for the purposes of the invention. "Amino acid" also includes imino acid residues such as proline and hydroxyproline. The side chains may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example  
10 to prevent or retard *in vivo* degradations. Chemical blocking groups or other chemical substituents may also be added.

In a preferred embodiment, the candidate bioactive agents are naturally occurring proteins or fragments of naturally occurring proteins. Thus, for example, cellular extracts containing proteins, or  
15 random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of procaryotic and eukaryotic proteins may be made for screening in the systems described herein. Particularly preferred in this embodiment are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred.

20 In a preferred embodiment, the candidate bioactive agents are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occurring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and  
25 amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below) are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized candidate bioactive proteinaceous agents.

30 In one embodiment, the library is fully randomized, with no sequence preferences or constants at any position. In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the nucleotides or amino acid residues are randomized within a defined class,  
35 for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

In a preferred embodiment, the candidate bioactive agents are nucleic acids. By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate

5 backbones, comprising, for example, phosphoramidate (Beaucage, *et al.*, Tetrahedron, **49**(10):1925 (1993) and references therein; Letsinger, *J. Org. Chem.*, **35**:3800 (1970); Sprinzl, *et al.*, Eur. J. Biochem., **81**:579 (1977); Letsinger, *et al.*, Nucl. Acids Res., **14**:3487 (1986); Sawai, *et al.*, Chem. Lett., 805 (1984); Letsinger, *et al.*, J. Am. Chem. Soc., **110**:4470 (1988); and Pauwels, *et al.*, Chemica Scripta, **26**:141 (1986)), phosphorothioate (Mag, *et al.*, Nucleic Acids Res., **19**:1437 (1991); and U.S.

10 Patent No. 5,644,048), phosphorodithioate (Briu, *et al.*, J. Am. Chem. Soc., **111**:2321 (1989)), O-methylphosphoramidite linkages (see Eckstein, *Oligonucleotides and Analogues: A Practical Approach*, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, *J. Am. Chem. Soc.*, **114**:1895 (1992); Meier, *et al.*, Chem. Int. Ed. Engl., **31**:1008 (1992); Nielsen, Nature, **365**:566 (1993); Carlsson, *et al.*, Nature, **380**:207 (1996), all of which are incorporated by

15 reference)). Other analog nucleic acids include those with positive backbones (Denpcy, *et al.*, Proc. Natl. Acad. Sci. USA, **92**:6097 (1995)); non-ionic backbones (U.S. Patent Nos. 5,386,023; 5,637,684; 5,602,240; 5,216,141; and 4,469,863; Kiedrowski, *et al.*, Angew. Chem. Intl. Ed. English, **30**:423 (1991); Letsinger, *et al.*, J. Am. Chem. Soc., **110**:4470 (1988); Letsinger, *et al.*, Nucleoside & Nucleotide, **13**:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate

20 Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker, *et al.*, Bioorganic & Medicinal Chem. Lett., **4**:395 (1994); Jeffs, *et al.*, J. Biomolecular NMR, **34**:17 (1994); Tetrahedron Lett., **37**:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing

25 one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins, *et al.*, Chem. Soc. Rev., (1995) pp. 169-176). Several nucleic acid analogs are described in Rawls, C & E News, June 2, 1997, page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules

30 in physiological environments. In addition, mixtures of naturally occurring nucleic acids and analogs can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the

35 nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine, isoguanine, etc.

As described above generally for proteins, nucleic acid candidate bioactive agents may be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of procaryotic or eukaryotic genomes may be used as is outlined above for proteins.

- 5 In a preferred embodiment, the candidate bioactive agents are organic chemical moieties, a wide variety of which are available in the literature.

- In a preferred embodiment, a library of different candidate bioactive agents are used. Preferably, the library should provide a sufficiently structurally diverse population of randomized agents to effect a
- 10 probabilistically sufficient range of diversity to allow binding to a particular target. Accordingly, an interaction library should be large enough so that at least one of its members will have a structure that gives it affinity for the target. Although it is difficult to gauge the required absolute size of an interaction library, nature provides a hint with the immune response: a diversity of  $10^7$ - $10^8$  different antibodies provides at least one combination with sufficient affinity to interact with most potential antigens
- 15 faced by an organism. Published *in vitro* selection techniques have also shown that a library size of  $10^7$  to  $10^8$  is sufficient to find structures with affinity for the target. A library of all combinations of a peptide 7 to 20 amino acids in length, such as generally proposed herein, has the potential to code for  $20^7$  ( $10^9$ ) to  $20^{20}$ . Thus, with libraries of  $10^7$  to  $10^8$  different molecules the present methods allow a "working" subset of a theoretically complete interaction library for 7 amino acids, and a subset of
- 20 shapes for the  $20^{20}$  library. Thus, in a preferred embodiment, at least  $10^6$ , preferably at least  $10^7$ , more preferably at least  $10^8$  and most preferably at least  $10^9$  different sequences are simultaneously analyzed in the subject methods. Preferred methods maximize library size and diversity.

- The candidate bioactive agents are combined or added to a cell or population of cells. Suitable cell
- 25 types for different embodiments are outlined above. The candidate bioactive agent and the cells are combined. As will be appreciated by those in the art, this may be accomplished in any number of ways, including adding the candidate agents to the surface of the cells, to the media containing the cells, or to a surface on which the cells are growing or in contact with; adding the agents into the cells, for example by using vectors that will introduce the agents into the cells (i.e. when the agents are nucleic
- 30 acids or proteins).

- In a preferred embodiment, the candidate bioactive agents are either nucleic acids or proteins (proteins in this context includes proteins, oligopeptides, and peptides) that are introduced into the host cells using vectors, including viral vectors. The choice of the vector, preferably a viral vector, will
- 35 depend on the cell type. When the cells are replicating, retroviral vectors are used as is more fully described below. When the cells are not replicating (i.e. they are arrested in one of the growth phases), other viral vectors may be used, including lentiviral and adenoviral vectors.

In a preferred embodiment, the cells are either replicating or can be induced to replicate, and retroviral vectors are used to introduce candidate bioactive agents to the cells, as is generally outlined in PCT US97/01019 and PCT US97/01048, both of which are expressly incorporated by reference. Generally, a library of retroviral vectors is made using retroviral packaging cell lines that are helper-defective and are capable of producing all the necessary trans proteins, including gag, pol and env, and RNA molecules that have in cis the  $\psi$  packaging signal. Briefly, the library is generated in a retrovirus DNA construct backbone; standard oligonucleotide synthesis is done to generate either the candidate agent or nucleic acid encoding a protein, for example a random peptide, using techniques well known in the art. After generation of the DNA library, the library is cloned into a first primer. The first primer serves as a "cassette", which is inserted into the retroviral construct. The first primer generally contains a number of elements, including for example, the required regulatory sequences (e.g. translation, transcription, promoters, etc), fusion partners, restriction endonuclease (cloning and subcloning) sites, stop codons (preferably in all three frames), regions of complementarity for second strand priming (preferably at the end of the stop codon region as minor deletions or insertions may occur in the random region), etc.

A second primer is then added, which generally consists of some or all of the complementarity region to prime the first primer and optional necessary sequences for a second unique restriction site for subcloning. DNA polymerase is added to make double-stranded oligonucleotides. The double-stranded oligonucleotides are cleaved with the appropriate subcloning restriction endonucleases and subcloned into the target retroviral vectors, described below.

Any number of suitable retroviral vectors may be used. Generally, the retroviral vectors may include: selectable marker genes as is more fully described below; promoters driving expression of a second gene, placed in sense or anti-sense relative to the 5' LTR; CRU5 (a synthetic LTR), tetracycline regulation elements in SIN, cell specific promoters, etc.

Preferred retroviral vectors include a vector based on the murine stem cell virus (MSCV) (see Hawley et al., Gene Therapy 1:136 (1994)) and a modified MFG virus (Riviere et al., Genetics 92:6733 (1995)), and pBABE, outlined in PCT US97/01019.

The retroviruses may include inducible and constitutive promoters for the expression of the candidate agent. For example, there are situations wherein it is necessary to induce peptide expression only during certain phases of the selection process, or only in certain cell phases (i.e. using phase specific promoters, such as E2F responsive promoter, p53 responsive promoter, cyclin promoters, etc.). A large number of both inducible and constitutive promoters are known.

In addition, it is possible to configure a retroviral vector to allow inducible expression of retroviral inserts after integration of a single-vector in target cells; importantly, the entire system is contained within the single retrovirus. Tet-inducible retroviruses have been designed incorporating the Self-Inactivating (SIN) feature of 3' LTR enhancer/promoter retroviral deletion mutant (Hoffman et al., PNAS USA 93:5185 (1996)). Expression of this vector in cells is virtually undetectable in the presence of tetracycline or other active analogs. However, in the absence of Tet, expression is turned on to maximum within 48 hours after induction, with uniform increased expression of the whole population of cells that harbor the inducible retrovirus, indicating that expression is regulated uniformly within the infected cell population. A similar, related system uses a mutated Tet DNA-binding domain such that it bound DNA in the presence of Tet, and was removed in the absence of Tet. Either of these systems is suitable.

In a preferred embodiment, the candidate bioactive agents are linked to a fusion partner. By "fusion partner" or "functional group" herein is meant a sequence that is associated with the candidate bioactive agent, that confers upon all members of the library in that class a common function or ability. Fusion partners can be heterologous (i.e. not native to the host cell), or synthetic (not native to any cell). Suitable fusion partners include, but are not limited to: a) presentation structures, as defined below, which provide the candidate bioactive agents in a conformationally restricted or stable form; b) targeting sequences, defined below, which allow the localization of the candidate bioactive agent into a subcellular or extracellular compartment; c) rescue sequences as defined below, which allow the purification or isolation of either the candidate bioactive agents or the nucleic acids encoding them; d) stability sequences, which confer stability or protection from degradation to the candidate bioactive agent or the nucleic acid encoding it, for example resistance to proteolytic degradation; e) dimerization sequences, to allow for peptide dimerization; or f) any combination of a), b), c), d), and e), as well as linker sequences as needed.

In a preferred embodiment, the fusion partner is a presentation structure. By "presentation structure" or grammatical equivalents herein is meant a sequence, which, when fused to candidate bioactive agents, causes the candidate agents to assume a conformationally restricted form. Proteins interact with each other largely through conformationally constrained domains. Although small peptides with freely rotating amino and carboxyl termini can have potent functions as is known in the art, the conversion of such peptide structures into pharmacologic agents is difficult due to the inability to predict side-chain positions for peptidomimetic synthesis. Therefore the presentation of peptides in conformationally constrained structures will benefit both the later generation of pharmaceuticals and will also likely lead to higher affinity interactions of the peptide with the target protein. This fact has been recognized in the combinatorial library generation systems using biologically generated short peptides in bacterial phage systems. A number of workers have constructed small domain molecules

in which one might present randomized peptide structures.

While the candidate bioactive agents may be either nucleic acid or peptides, presentation structures are preferably used with peptide candidate agents. Thus, synthetic presentation structures, i.e.

- 5 artificial polypeptides, are capable of presenting a randomized peptide as a conformationally-restricted domain. Generally such presentation structures comprise a first portion joined to the N-terminal end of the randomized peptide, and a second portion joined to the C-terminal end of the peptide; that is, the peptide is inserted into the presentation structure, although variations may be made, as outlined below. To increase the functional isolation of the randomized expression product, the presentation  
10 structures are selected or designed to have minimal biological activity when expressed in the target cell.

- Preferred presentation structures maximize accessibility to the peptide by presenting it on an exterior loop. Accordingly, suitable presentation structures include, but are not limited to, minibody structures,  
15 loops on beta-sheet turns and coiled-coil stem structures in which residues not critical to structure are randomized, zinc-finger domains, cysteine-linked (disulfide) structures, transglutaminase linked structures, cyclic peptides, B-loop structures, helical barrels or bundles, leucine zipper motifs, etc.

- In a preferred embodiment, the presentation structure is a coiled-coil structure, allowing the  
20 presentation of the randomized peptide on an exterior loop. See, for example, Myszkowski et al., Biochem. 33:2362-2373 (1994), hereby incorporated by reference). Using this system investigators have isolated peptides capable of high affinity interaction with the appropriate target. In general, coiled-coil structures allow for between 6 to 20 randomized positions.

- 25 A preferred coiled-coil presentation structure is as follows:  
**MGCA**ALESEVSALESEVASLESEVAAL**GRGDMP**LAAVKSKLSAVKSKLASVKSKLAA**CGPP**. The underlined regions represent a coiled-coil leucine zipper region defined previously (see Martin et al., EMBO J. 13(22):5303-5309 (1994), incorporated by reference). The bolded GRGDMP region represents the loop structure and when appropriately replaced with randomized peptides (i.e.  
30 candidate bioactive agents, generally depicted herein as  $(X)_n$ , where X is an amino acid residue and n is an integer of at least 5 or 6) can be of variable length. The replacement of the bolded region is facilitated by encoding restriction endonuclease sites in the underlined regions, which allows the direct incorporation of randomized oligonucleotides at these positions. For example, a preferred embodiment generates a XhoI site at the double underlined LE site and a HindIII site at the double-  
35 underlined KL site.

In a preferred embodiment, the presentation structure is a minibody structure. A "minibody" is



essentially composed of a minimal antibody complementarity region. The minibody presentation structure generally provides two randomizing regions that in the folded protein are presented along a single face of the tertiary structure. See for example Bianchi et al., J. Mol. Biol. 236(2):649-59 (1994), and references cited therein, all of which are incorporated by reference). Investigators have shown  
 5 this minimal domain is stable in solution and have used phage selection systems in combinatorial libraries to select minibodies with peptide regions exhibiting high affinity,  $K_d = 10^{-7}$ , for the pro-inflammatory cytokine IL-6.

A preferred minibody presentation structure is as follows:

10 **MGRNSQATSGFTFSHEYMEWVRGGEYIAASRHKHNKYTTEYSASVKGRYIVSRDTSQSILYLQKKKG**  
**PP.** The bold, underline regions are the regions which may be randomized. The italicized phenylalanine must be invariant in the first randomizing region. The entire peptide is cloned in a three-oligonucleotide variation of the coiled-coil embodiment, thus allowing two different randomizing regions to be incorporated simultaneously. This embodiment utilizes non-palindromic BstXI sites on the termini.

15 In a preferred embodiment, the presentation structure is a sequence that contains generally two cysteine residues, such that a disulfide bond may be formed, resulting in a conformationally constrained sequence. This embodiment is particularly preferred when secretory targeting sequences are used. As will be appreciated by those in the art, any number of random sequences, with or without  
 20 spacer or linking sequences, may be flanked with cysteine residues. In other embodiments, effective presentation structures may be generated by the random regions themselves. For example, the random regions may be "doped" with cysteine residues which, under the appropriate redox conditions, may result in highly crosslinked structured conformations, similar to a presentation structure. Similarly, the randomization regions may be controlled to contain a certain number of residues to confer  $\beta$ -sheet  
 25 or  $\alpha$ -helical structures.

In a preferred embodiment, the fusion partner is a targeting sequence. As will be appreciated by those in the art, the localization of proteins within a cell is a simple method for increasing effective concentration and determining function. For example, RAF1 when localized to the mitochondrial  
 30 membrane can inhibit the anti-apoptotic effect of BCL-2. Similarly, membrane bound Sos induces Ras mediated signaling in T-lymphocytes. These mechanisms are thought to rely on the principle of limiting the search space for ligands, that is to say, the localization of a protein to the plasma membrane limits the search for its ligand to that limited dimensional space near the membrane as opposed to the three dimensional space of the cytoplasm. Alternatively, the concentration of a protein can also be simply  
 35 increased by nature of the localization. Shuttling the proteins into the nucleus confines them to a smaller space thereby increasing concentration. Finally, the ligand or target may simply be localized to a specific compartment, and inhibitors must be localized appropriately.

Thus, suitable targeting sequences include, but are not limited to, binding sequences capable of causing binding of the expression product to a predetermined molecule or class of molecules while retaining bioactivity of the expression product, (for example by using enzyme inhibitor or substrate sequences to target a class of relevant enzymes); sequences signalling selective degradation, of itself  
 5 or co-bound proteins; and signal sequences capable of constitutively localizing the candidate expression products to a predetermined cellular locale, including a) subcellular locations such as the Golgi, endoplasmic reticulum, nucleus, nucleoli, nuclear membrane, mitochondria, chloroplast, secretory vesicles, lysosome, and cellular membrane; and b) extracellular locations via a secretory signal. Particularly preferred is localization to either subcellular locations or to the outside of the cell  
 10 via secretion.

In a preferred embodiment, the targeting sequence is a nuclear localization signal (NLS). NLSs are generally short, positively charged (basic) domains that serve to direct the entire protein in which they occur to the cell's nucleus. Numerous NLS amino acid sequences have been reported including single  
 15 basic NLS's such as that of the SV40 (monkey virus) large T Antigen (Pro Lys Lys Lys Arg Lys Val), Kalderon (1984), et al., Cell, 39:499-509; the human retinoic acid receptor- $\beta$  nuclear localization signal (ARRRRP); NF $\kappa$ B p50 (EEVQRKRQKL; Ghosh et al., Cell 62:1019 (1990); NF $\kappa$ B p65 (EEKRKRTYE; Nolan et al., Cell 64:961 (1991); and others (see for example Boulikas, J. Cell. Biochem. 55(1):32-58 (1994), hereby incorporated by reference) and double basic NLS's exemplified by that of the Xenopus  
 20 (African clawed toad) protein, nucleoplasmin (Ala Val Lys Arg Pro Ala Ala Thr Lys Lys Ala Gly Gln Ala Lys Lys Lys Lys Leu Asp), Dingwall, et al., Cell, 30:449-458, 1982 and Dingwall, et al., J. Cell Biol., 107:641-849; 1988). Numerous localization studies have demonstrated that NLSs incorporated in synthetic peptides or grafted onto reporter proteins not normally targeted to the cell nucleus cause these peptides and reporter proteins to be concentrated in the nucleus. See, for example, Dingwall,  
 25 and Laskey, Ann. Rev. Cell Biol., 2:367-390, 1986; Bonnerot, et al., Proc. Natl. Acad. Sci. USA, 84:6795-6799, 1987; Galileo, et al., Proc. Natl. Acad. Sci. USA, 87:458-462, 1990.

In a preferred embodiment, the targeting sequence is a membrane anchoring signal sequence. This is particularly useful since many parasites and pathogens bind to the membrane, in addition to the fact  
 30 that many intracellular events originate at the plasma membrane. Thus, membrane-bound peptide libraries are useful for both the identification of important elements in these processes as well as for the discovery of effective inhibitors. The invention provides methods for presenting the randomized expression product extracellularly or in the cytoplasmic space; see Fig 3. For extracellular presentation, a membrane anchoring region is provided at the carboxyl terminus of the peptide  
 35 presentation structure. The randomized expression product region is expressed on the cell surface and presented to the extracellular space, such that it can bind to other surface molecules (affecting their function) or molecules present in the extracellular medium. The binding of such molecules could

- confer function on the cells expressing a peptide that binds the molecule. The cytoplasmic region could be neutral or could contain a domain that, when the extracellular randomized expression product region is bound, confers a function on the cells (activation of a kinase, phosphatase, binding of other cellular components to effect function). Similarly, the randomized expression product-containing
- 5 region could be contained within a cytoplasmic region, and the transmembrane region and extracellular region remain constant or have a defined function.

- Membrane-anchoring sequences are well known in the art and are based on the genetic geometry of mammalian transmembrane molecules. Peptides are inserted into the membrane based on a signal
- 10 sequence (designated herein as ssTM) and require a hydrophobic transmembrane domain (herein TM). The transmembrane proteins are inserted into the membrane such that the regions encoded 5' of the transmembrane domain are extracellular and the sequences 3' become intracellular. Of course, if these transmembrane domains are placed 5' of the variable region, they will serve to anchor it as an intracellular domain, which may be desirable in some embodiments. ssTMs and TMs are known for a
- 15 wide variety of membrane bound proteins, and these sequences may be used accordingly, either as pairs from a particular protein or with each component being taken from a different protein, or alternatively, the sequences may be synthetic, and derived entirely from consensus as artificial delivery domains.

- 20 As will be appreciated by those in the art, membrane-anchoring sequences, including both ssTM and TM, are known for a wide variety of proteins and any of these may be used. Particularly preferred membrane-anchoring sequences include, but are not limited to, those derived from CD8, ICAM-2, IL-8R, CD4 and LFA-1.
- 25 Useful sequences include sequences from: 1) class I integral membrane proteins such as IL-2 receptor beta-chain (residues 1-26 are the signal sequence, 241-265 are the transmembrane residues; see Hatakeyama et al., Science 244:551 (1989) and von Heijne et al, Eur. J. Biochem. 174:671 (1988)) and insulin receptor beta chain (residues 1-27 are the signal, 957-959 are the transmembrane domain and 960-1382 are the cytoplasmic domain; see Hatakeyama, supra, and
- 30 Ebina et al., Cell 40:747 (1985)); 2) class II integral membrane proteins such as neutral endopeptidase (residues 29-51 are the transmembrane domain, 2-28 are the cytoplasmic domain; see Malfroy et al., Biochem. Biophys. Res. Commun. 144:59 (1987)); 3) type III proteins such as human cytochrome P450 NF25 (Hatakeyama, supra); and 4) type IV proteins such as human P-glycoprotein (Hatakeyama, supra). Particularly preferred are CD8 and ICAM-2. For example, the signal
- 35 sequences from CD8 and ICAM-2 lie at the extreme 5' end of the transcript. These consist of the amino acids 1-32 in the case of CD8 (MASPLTRFLSLNLLLLGESILGSGEAKPQAP; Nakauchi et al., PNAS USA 82:5126 (1985) and 1-21 in the case of ICAM-2 (MSSFGYRTLTVALTLCICPG; Staunton

et al., Nature (London) 339:61 (1989)). These leader sequences deliver the construct to the membrane while the hydrophobic transmembrane domains, placed 3' of the random candidate region, serve to anchor the construct in the membrane. These transmembrane domains are encompassed by amino acids 145-195 from CD8

- 5 (PQRPEDCRPRGSKGTGLDFACDIYWAPLAGICVALLSLIITLICYHSR; Nakauchi, supra) and 224-256 from ICAM-2 (MVIIVTVVSVLLSLFVTSVLLCFIFGQHRLRQQR; Staunton, supra).

- Alternatively, membrane anchoring sequences include the GPI anchor, which results in a covalent bond between the molecule and the lipid bilayer via a glycosyl-phosphatidylinositol bond for example  
10 in DAF (PNKGS~~GT~~TS~~GT~~TRLLSGHTCFTLTGLLGTLVTMGLLT, with the bolded serine the site of the anchor; see Homans et al., Nature 333(6170):269-72 (1988), and Moran et al., J. Biol. Chem. 266:1250 (1991)). In order to do this, the GPI sequence from Thy-1 can be cassetted 3' of the variable region in place of a transmembrane sequence.

- 15 Similarly, myristylation sequences can serve as membrane anchoring sequences. It is known that the myristylation of c-src recruits it to the plasma membrane. This is a simple and effective method of membrane localization, given that the first 14 amino acids of the protein are solely responsible for this function: MGSSKSKPKDPSQR (see Cross et al., Mol. Cell. Biol. 4(9):1834 (1984); Spencer et al., Science 262:1019-1024 (1993), both of which are hereby incorporated by reference). This motif has  
20 already been shown to be effective in the localization of reporter genes and can be used to anchor the zeta chain of the TCR. This motif is placed 5' of the variable region in order to localize the construct to the plasma membrane. Other modifications such as palmitoylation can be used to anchor constructs in the plasma membrane; for example, palmitoylation sequences from the G protein-coupled receptor kinase GRK6 sequence (LLQRLFSRQDCCGNCSDSEELPTRL, with the bold cysteines being  
25 palmitoylated; Stoffel et al., J. Biol. Chem 269:27791 (1994)); from rhodopsin (KQFRNCMLTSLCCGKNPLGD; Barnstable et al., J. Mol. Neurosci. 5(3):207 (1994)); and the p21 H-ras 1 protein (LNPPDESGPGCMSCKCVLS; Capon et al., Nature 302:33 (1983)).

- In a preferred embodiment, the targeting sequence is a lysosomal targeting sequence, including, for  
30 example, a lysosomal degradation sequence such as Lamp-2 (KFERQ; Dice, Ann. N.Y. Acad. Sci. 674:58 (1992); or lysosomal membrane sequences from Lamp-1 (MLIPIAGFFALAGLVLI~~VLI~~AYLIGRKRSHAGYQTI, Uthayakumar et al., Cell. Mol. Biol. Res. 41:405 (1995)) or Lamp-2 (LVPIAVGAALAGVLILVLLAYFIGLKHHHAGYEQE, Konecki et al., Biochem. Biophys. Res. Comm. 205:1-5 (1994), both of which show the transmembrane domains in italics and  
35 the cytoplasmic targeting signal underlined).

Alternatively, the targeting sequence may be a mitochondrial localization sequence, including

- mitochondrial matrix sequences (e.g. yeast alcohol dehydrogenase III; MLRTSSLFTRRVQPSLFSRNILRLQST; Schatz, Eur. J. Biochem. 165:1-6 (1987)); mitochondrial inner membrane sequences (yeast cytochrome c oxidase subunit IV; MLSLRQSIRFFKPATRTLCSRYLL; Schatz, supra); mitochondrial intermembrane space sequences (yeast cytochrome c1; 5 MFSMLSKRWAQRTLKSFYSTATGAASKSGKLTQKLVTAGVAAAGITASTLLYADSLTAEAMTA; Schatz, supra) or mitochondrial outer membrane sequences (yeast 70 kD outer membrane protein; MKSFITRNKTALATVAATGTAIGAYYYNQLQQQQQRGKK; Schatz, supra).

- The target sequences may also be endoplasmic reticulum sequences, including the sequences from 10 calreticulin (KDEL; Pelham, Royal Society London Transactions B; 1-10 (1992)) or adenovirus E3/19K protein (LYLSRRSFIDEKKMP; Jackson et al., EMBO J. 9:3153 (1990)).

- Furthermore, targeting sequences also include peroxisome sequences (for example, the peroxisome matrix sequence from Luciferase; SKL; Keller et al., PNAS USA 4:3264 (1987)); farnesylation 15 sequences (for example, P21 H-ras 1; LNPPDESGPGCMSCKCVLS, with the bold cysteine farnesylated; Capon, supra); geranylgeranylation sequences (for example, protein rab-5A; LTEPTQPTRNQCCSN, with the bold cysteines geranylgeranylated; Farnsworth, PNAS USA 91:11963 (1994)); or destruction sequences (cyclin B1; RTALGDIGN; Klotzbucher et al., EMBO J. 1:3053 (1996)).

- 20 In a preferred embodiment, the targeting sequence is a secretory signal sequence capable of effecting the secretion of the candidate translation product. There are a large number of known secretory signal sequences which are placed 5' to the variable peptide region, and are cleaved from the peptide region to effect secretion into the extracellular space. Secretory signal sequences and their transferability to 25 unrelated proteins are well known, e.g., Silhavy, et al. (1985) Microbiol. Rev. 49, 398-418. This is particularly useful to generate a peptide capable of binding to the surface of, or affecting the physiology of, a target cell that is other than the host cell, e.g., the cell expressing the peptide. In a preferred approach, a fusion product is configured to contain, in series, secretion signal peptide-presentation structure-randomized expression product region-presentation structure. In this manner, 30 target cells grown in the vicinity of cells caused to express the library of peptides, are bathed in secreted peptide. Target cells exhibiting a physiological change in response to the presence of a peptide, e.g., by the peptide binding to a surface receptor or by being internalized and binding to intracellular targets, and the secreting cells are localized by any of a variety of selection schemes and the peptide causing the effect determined. Exemplary effects include variously that of a designer 35 cytokine (i.e., a stem cell factor capable of causing hematopoietic stem cells to divide and maintain their totipotential), a factor causing cancer cells to undergo spontaneous apoptosis, a factor that binds to the cell surface of target cells and labels them specifically, etc.

- Suitable secretory sequences are known, including signals from IL-2 (MYRMQLLSICIALSLALVTNS; Villinger et al., J. Immunol. 155:3946 (1995)), growth hormone (MATGSRTSLLLAFLGLCLPWLQEGSAFPT; Roskam et al., Nucleic Acids Res. 7:30 (1979)); preproinsulin (MALWMRLLPLLALLALWGPDPAAAEVN; Bell et al., Nature 284:26 (1980)); and
- 5 influenza HA protein (MKAKLLVLLYAFVAGDQI; Sekiwawa et al., PNAS 80:3563)), with cleavage between the non-underlined-underlined junction. A particularly preferred secretory signal sequence is the signal leader sequence from the secreted cytokine IL-4, which comprises the first 24 amino acids of IL-4 as follows: MGLTSQLLPPLFFLLACAGNFVHG.
- 10 In a preferred embodiment, the fusion partner is a rescue sequence. A rescue sequence is a sequence which may be used to purify or isolate either the candidate agent or the nucleic acid encoding it. Thus, for example, peptide rescue sequences include purification sequences such as the His<sub>6</sub> tag for use with Ni affinity columns and epitope tags for detection, immunoprecipitation or FACS (fluorescence-activated cell sorting). Suitable epitope tags include myc (for use with the commercially
- 15 available 9E10 antibody), the BSP biotinylation target sequence of the bacterial enzyme BirA, flu tags, lacZ, and GST.

- Alternatively, the rescue sequence may be a unique oligonucleotide sequence which serves as a probe target site to allow the quick and easy isolation of the retroviral construct, via PCR, related
- 20 techniques, or hybridization.

- In a preferred embodiment, the fusion partner is a stability sequence to confer stability to the candidate bioactive agent or the nucleic acid encoding it. Thus, for example, peptides may be stabilized by the incorporation of glycines after the initiation methionine (MG or MGG0), for protection of the peptide to
- 25 ubiquitination as per Varshavsky's N-End Rule, thus conferring long half-life in the cytoplasm. Similarly, two prolines at the C-terminus impart peptides that are largely resistant to carboxypeptidase action. The presence of two glycines prior to the prolines impart both flexibility and prevent structure initiating events in the di-proline to be propagated into the candidate peptide structure. Thus, preferred stability sequences are as follows: MG(X)<sub>n</sub>GGPP, where X is any amino acid and n is an integer of at
- 30 least four.

- In one embodiment, the fusion partner is a dimerization sequence. A dimerization sequence allows the non-covalent association of one random peptide to another random peptide, with sufficient affinity to remain associated under normal physiological conditions. This effectively allows small libraries of
- 35 random peptides (for example, 10<sup>4</sup>) to become large libraries if two peptides per cell are generated which then dimerize, to form an effective library of 10<sup>8</sup> (10<sup>4</sup> X 10<sup>4</sup>). It also allows the formation of longer random peptides, if needed, or more structurally complex random peptide molecules. The

dimers may be homo- or heterodimers.

Dimerization sequences may be a single sequence that self-aggregates, or two sequences, each of which is generated in a different retroviral construct. That is, nucleic acids encoding both a first  
5 random peptide with dimerization sequence 1, and a second random peptide with dimerization sequence 2, such that upon introduction into a cell and expression of the nucleic acid, dimerization sequence 1 associates with dimerization sequence 2 to form a new random peptide structure.

Suitable dimerization sequences will encompass a wide variety of sequences. Any number of protein-  
10 protein interaction sites are known. In addition, dimerization sequences may also be elucidated using standard methods such as the yeast two hybrid system, traditional biochemical affinity binding studies, or even using the present methods.

The fusion partners may be placed anywhere (i.e. N-terminal, C-terminal, internal) in the structure as  
15 the biology and activity permits.

In a preferred embodiment, the fusion partner includes a linker or tethering sequence, as generally described in PCT US 97/01019, that can allow the candidate agents to interact with potential targets unhindered. For example, when the candidate bioactive agent is a peptide, useful linkers include  
20 glycine-serine polymers (including, for example,  $(GS)_n$ ,  $(GSGGS)_n$  and  $(GGGS)_n$ , where  $n$  is an integer of at least one), glycine-alanine polymers, alanine-serine polymers, and other flexible linkers such as the tether for the shaker potassium channel, and a large variety of other flexible linkers, as will be appreciated by those in the art. Glycine-serine polymers are preferred since both of these amino acids are relatively unstructured, and therefore may be able to serve as a neutral tether between  
25 components. Secondly, serine is hydrophilic and therefore able to solubilize what could be a globular glycine chain. Third, similar chains have been shown to be effective in joining subunits of recombinant proteins such as single chain antibodies.

In addition, the fusion partners, including presentation structures, may be modified, randomized,  
30 and/or matured to alter the presentation orientation of the randomized expression product. For example, determinants at the base of the loop may be modified to slightly modify the internal loop peptide tertiary structure, which maintaining the randomized amino acid sequence.

In a preferred embodiment, combinations of fusion partners are used. Thus, for example, any number  
35 of combinations of presentation structures, targeting sequences, rescue sequences, and stability sequences may be used, with or without linker sequences.

Thus, candidate agents can include these components, and may then be used to generate a library of fragments, each containing a different random nucleotide sequence that may encode a different peptide. The ligation products are then transformed into bacteria, such as *E. coli*, and DNA is prepared from the resulting library, as is generally outlined in Kitamura, PNAS USA 92:9146-9150 (1995), hereby expressly incorporated by reference.

Delivery of the library DNA into a retroviral packaging system results in conversion to infectious virus. Suitable retroviral packaging system cell lines include, but are not limited to, the Bing and BOSC23 cell lines described in WO 94/19478; Soneoka et al., Nucleic Acid Res. 23(4):628 (1995); Finer et al., Blood 83:43 (1994); Phoenix packaging lines such as PhiNX-eco and PhiNX-ampho, described below; 292T + gag-pol and retrovirus envelope; PA317; and cell lines outlined in Markowitz et al., Virology 167:400 (1988), Markowitz et al., J. Virol. 62:1120 (1988), Li et al., PNAS USA 93:11658 (1996), Kinsella et al., Human Gene Therapy 7:1405 (1996), all of which are incorporated by reference. Preferred systems include PhiNX-eco and PhiNX-ampho or similar cell lines, disclosed in PCT US97/01019.

When the cells are not replicating, other viral vectors may be used, including adenoviral vectors, feline immunoviral (FIV) vectors, etc.

In a preferred embodiment, when the candidate agent is introduced to the cells using a viral vector, the candidate peptide agent is linked to a detectable molecule, and the methods of the invention include at least one expression assay. An expression assay is an assay that allows the determination of whether a candidate bioactive agent has been expressed, i.e. whether a candidate peptide agent is present in the cell. Thus, by linking the expression of a candidate agent to the expression of a detectable molecule such as a label, the presence or absence of the candidate peptide agent may be determined. Accordingly, in this embodiment, the candidate agent is operably linked to a detectable molecule. Generally, this is done by creating a fusion nucleic acid. The fusion nucleic acid comprises a first nucleic acid encoding the candidate bioactive agent (which can include fusion partners, as outlined above), and a second nucleic acid encoding a detectable molecule. The terms "first" and "second" are not meant to confer an orientation of the sequences with respect to 5'-3' orientation of the fusion nucleic acid. For example, assuming a 5'-3' orientation of the fusion sequence, the first nucleic acid may be located either 5' to the second nucleic acid, or 3' to the second nucleic acid. Preferred detectable molecules in this embodiment include, but are not limited to, fluorescent proteins, including GFP, YFP, BFP and RFP, with the former being especially preferred.

In general, the candidate agents are added to the cells (either extracellularly or intracellularly, as outlined above) under reaction conditions that favor agent-target interactions. Generally, this will be



physiological conditions. Incubations may be performed at any temperature which facilitates optimal activity, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high through put screening. Typically between 0.1 and 1 hour will be sufficient. Excess reagent is generally removed or washed away.

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A variety of other reagents may be included in the assays. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc.,

- 10 may be used. The mixture of components may be added in any order that provides for detection. Washing or rinsing the cells will be done as will be appreciated by those in the art at different times, and may include the use of filtration and centrifugation. When second labeling moieties (also referred to herein as "secondary labels") are used, they are preferably added after excess non-bound target molecules are removed, in order to reduce non-specific binding; however, under some circumstances,
- 15 all the components may be added simultaneously.

- In a preferred embodiment, the cells are sorted using fluorescent-activated cell sorting (FACS). In the invention herein, cell cycle regulation is evaluated by multiple parameters which results in reduced background and greater specificity. In contrast, FACS has been used in the past to evaluate two
- 20 different or unrelated characteristics at the same time which identifies cells having those two characteristics, but does not reduce the background for the combined characteristics.

- Thus, the cells are sorted or enriched in a FACS on the basis of one or more of the assays, including a cell viability assay, a proliferation assay, a cell phase assay, and (when candidate agents are
- 25 expressed with detectable moieties) an expression assay. The results from one or more of these assays are compared to cells that were not exposed to the candidate bioactive agent, or to the same cells prior to introduction of the candidate agent. Alterations in these results can indicate that said agent modulates cell cycle regulation.

- 30 A strength of the present invention is that a library of candidate agents may be tested in a library of cells, because the present methods allow single cell sorting, with extremely high specificity, such that very rare events may be detected. The use of multiple laser paths allows sort accuracy of 1 in  $10^6$  with better than 70% accuracy.

- 35 In addition, the present invention can, in addition to the identification of multiple cell cycle regulation properties, be combined with the identification of other cellular characteristics. For example, parameters of general cellular health can be determined and selected for by using i.e., dye Indo-1

indicating a calcium response. Other cellular parameters which are routinely identified by the skilled artisan include but are not limited to: cell size, cell shape, redox state, DNA content, nucleic acid sequence, chromatin structure, RNA content, total protein, antigens, lipids, surface proteins, intracellular receptors, oxidative metabolism, DNA synthesis and degradation and intracellular pH.

5

In a preferred embodiment, each of the measurements is determined simultaneously from an individual cell as it passes through the beam paths of multiple lasers. Alternatively, the measurements are done sequentially. By using more than one parameter to detect cell cycle regulation or alterations in cell cycle regulation, background is reduced and specificity is increased. The cells meeting the parameters of the desired properties can be physically sorted from cells not meeting the desired parameters or they can be identified by their percentage in the cell population.

In general,  $K_D$ s of  $\leq 1 \mu M$  are preferred, to allow for retention of binding in the presence of the shear forces present in FACS sorting. In a preferred embodiment, the cells are sorted at very high speeds, for example greater than about 5,000 sorting events per sec, with greater than about 10,000 sorting events per sec being preferred, and greater than about 25,000 sorting events per second being particularly preferred, with speeds of greater than about 50,000 to 100,000 being especially preferred.

Cells processed for stimulation and staining are generally taken up in buffer and filtered prior to cytometry. Cells can be analyzed using a FACSCAN (Becton Dickinson Inc., laser line 488nm) or a Mo-Flo (Cytomation, Inc., laser lines 350nm broadband (UV), 488nm, and 647nm) Cytometer. Cells are sorted, if desired, using the Mo-Flo.

Wherein the cells are analyzed by microscopy, cells post stimulation or staining are generally mounted onto glass slides and coverslipped; these are directly visualized by brightfield and fluorescence microscopy on an inverted microscope (i.e., TE300, Nikon) using standard BFP, FITC, or TRITC (for example) filter sets. Images can also be obtained using an inverted confocal scanning microscope (Zeiss, Inc., Bio-Rad, Inc.) using standard FITC and TRITC (for example) filter sets.

The sorting results in a population of cells having the desired properties. In a preferred embodiment, the parameters are set to identify at least one candidate bioactive agent that modulates cell cycle regulation.

In a preferred embodiment, the bioactive agent is characterized. This will proceed as will be appreciated by those in the art, and generally includes an analysis of the structure, identity, binding affinity and function of the agent. Generally, once identified, the bioactive agent is resynthesized and combined with the target cell to verify the cell cycle regulation modulation under various conditions

and in the presence or absence of other various agents. The bioactive can be prepared in a therapeutically effective amount to modulate cell cycle regulation and combined with a suitable pharmaceutical carrier.

- 5 In a preferred embodiment, the cell populations can be subjected to various experimental conditions, with and without the candidate agents. Changes in conditions include but are not limited to changes in pH, temperature, buffer or salt concentration, etc. In a preferred embodiment, the pH is changed, generally by increasing or decreasing the pH, usually by from about 0.5 to about 3 pH units. Alternatively, the temperature is altered, with increases or decreases of from about 5°C to about 30 °C
- 10 being preferred. Similarly, the salt concentration may be modified, with increases or decreases of from about 0.1 M to about 2 M being preferred.

- It is understood by the skilled artisan that the steps of the assays provided herein can vary in order. It is also understood, however, that while various options (of compounds, properties selected or order of
- 15 steps) are provided herein, the options are also each provided individually, and can each be individually segregated from the other options provided herein. Moreover, steps which are obvious and known in the art that will increase the sensitivity of the assay are intended to be within the scope of this invention. For example, there may be additionally washing steps, or segregation, isolation steps. Moreover, it is understood that in some cases detection is in the cells, but can also take place
- 20 in the media, or vice versa.

- In a preferred embodiment, the cellular phenotype is exocytosis, and the methods and compositions of the invention are directed to the detection of alterations in exocytosis, again using a FACS machine. There are a number of parameters that may be evaluated or assayed to allow the detection of
- 25 alterations in exocytotic pathways, including, but not limited to, light scattering, fluorescent dye uptake, fluorescent dye release, granule exposure, surface granule enzyme activity, and the quantity of granule specific proteins. By assaying or measuring one or more of these parameters, it is possible to detect not only alterations in exocytosis, but alterations of different steps of the exocytotic pathway. In addition, multiparameter analysis also reduces the background, or "false positives", that are detected.
- 30 In this manner, rapid, accurate screening of candidate agents may be performed to identify agents that modulate exocytosis.

- In a preferred embodiment, the invention provides methods for screening for alterations in exocytosis of a population of cells. By "alteration" or "modulation" in the context of exocytosis is meant a
- 35 decrease or an increase in the amount of exocytosis in one cell compared to another cell or in the same cell under different conditions. The measurements can be determined wherein all of the conditions are the same for each measurement, or under various conditions, with or without bioactive

agents, or at different stages of the exocytic process. For example, a measurement of exocytosis can be determined in a cell population wherein a candidate bioactive agent is present and wherein the candidate bioactive agent is absent. In another example, the measurements of exocytosis are determined wherein the condition or environment of the populations of cells differ from one another.

- 5 For example, the cells may be evaluated in the presence or absence of physiological signals, such as exocytic inducers (i.e.,  $\text{Ca}^{++}$ , ionomycin, etc.), hormones, antibodies, peptides, antigens, cytokines, growth factors, action potentials, or other cells (i.e. cell-cell contacts). In another example, the measurements of exocytosis are determined at different stages of the exocytic process. In yet another example, the measurements of exocytosis are taken wherein the conditions are the same, and the
- 10 alterations are between one cell or cell population and another cell or cell population.

- By a "population of cells" herein is meant a sample of cells as defined above. In this embodiment, the cells are preferably (but not required) to be rapidly growing, retrovirally infectable, and compatible with dyes and antibodies. Preferred cell types for use in this embodiment, include, but are not limited to,
- 15 mast cells, neurons, adrenal chromaffin cells, basophils, endocrine cells including pancreatic  $\beta$ -cells, pancreatic acinar cells including exocrine cells, neutrophils, monocytes, lymphocytes, mammary cells, sperm, egg cells and PMN leukocytes, endothelial cells, adipocytes, and muscle cells.

- The exocytotic methods comprise sorting the cells in a FACS machine by assaying for alterations in at
- 20 least three of the properties selected from the group consisting of light scattering, fluorescent dye uptake, fluorescent dye release, granule exposure, surface granule enzyme activity, and the quantity of granule specific proteins. In a preferred embodiment, each of the measurements is determined simultaneously from an individual cell as it passes through the beam paths of multiple lasers. Alternatively, the measurements are done sequentially. By using more than one parameter to detect
- 25 exocytosis or alterations in exocytosis, background is reduced and specificity is increased. The cells meeting the parameters of the desired properties can be physically sorted from cells not meeting the desired parameters or they can be identified by their percentage in the cell population.

- In a preferred embodiment, changes in light scattering are assayed to determine alterations in
- 30 exocytosis in a population of cells. When viewed in the FACS, cells have particular characteristics as measured by their forward and 90 degree (side) light scatter properties. These scatter properties represent the size, shape and granule content of the cells. Upon activation of the cells with a pro-exocytic stimulus, both the forward and side scatter properties of the cells changes considerably. These properties account for two parameters to be measured as a readout for the exocytic event.
- 35 These properties change in proportion to the extent of exocytosis of the cells and depend on the time course of the exocytic events as well. Alterations in the intensity of light scattering, or the cell-refractive index indicate alterations in exocytosis either in the same cell at different times, or compared

to the same cell under different conditions or with candidate bioactive agents present or absent, or compared to different cells or cell populations.

- In one embodiment provided herein, a cell population is combined with an agent which is known to stimulate exocytosis and the light scattering properties are determined. Cells having light scattering properties indicating the desirable exocytic activity can be identified and/or sorted. Exocytic activity as used herein includes lack of activity. In a preferred embodiment, candidate bioactive agents are combined with the cell population prior to or with the exocytic stimulus, as is more fully outlined below. In this embodiment, where light scattering properties differ as between a) a cell population combined with a known exocytic stimulus and a candidate bioactive agent, and b) a cell population combined with a known exocytic stimulus wherein the candidate bioactive agent is absent, it can be determined that the candidate bioactive agent modulates exocytosis. It may also be desirable in some cases to include an inhibitor of exocytosis or to exclude the exocytic stimulus to identify bioactive agents which induce exocytosis. Preferably, light scattering properties are measured in combination with at least one, and preferably two other properties which indicate exocytosis activity. General methodologies for light scattering measurements are further described in Perretti, et al., J. Pharmacol. Methods, 23(3):187-194 (1990) and Hide et al., J. Cell Biol., 123(3):585-593 (1993), both incorporated herein by reference. In general, changes of at least about 5% from baseline are preferred, with at least about 25% being more preferred, at least about 50% being particularly preferred, and at least about 75 to 100% being especially preferred. Baseline in this case generally means the light scatter properties of the cells prior to exocytotic stimulation. In each case provided herein, the baseline may also be set for any control parameter. For example, the baseline may be set at the exocytosis measurement of a particular cell, a similar cell under different conditions, or at a particular time point during exocytosis.
- In another preferred embodiment, changes in fluorescent dye uptake are evaluated. Preferred fluorescent dyes include styryl dyes, which indicate exocytosis activity in relation to endocytosis, sometimes referred to as coupled endocytosis. The theory behind coupled endocytosis is that cells undergoing exocytosis must also undergo endocytosis in order to maintain cell volume and membrane integrity. Thus, upon exocytic stimulation, endocytosis is also increased, providing an indirect measurement of exocytosis by quantifying the amount of styryl dye uptake.

- In an embodiment provided herein, the cells are bathed in a solution of styryl dye and stimulated with a pro-exocytic stimulus and the dye is quantitated. Preferably, after exocytic stimulation, the cells are spun down, aspirated and resuspended in fresh buffer. In a preferred embodiment, a candidate bioactive agent is combined with the cells as described herein. In some cases, the candidate bioactive agent can be combined with the cells with an inhibitor of exocytosis or without the pro-exocytic stimulus. Preferably, a pro-exocytic stimulus is added to the cell population which results in a

- dramatic increase in the fluorescence signal of the dye. The increased cell associated signal is due to coupled endocytosis of the styryl dye and is proportional to the exocytic response in both time and intensity. Conversely, the signal is not increased wherein exocytosis is inhibited or is not induced. Alterations are determined by measuring the fluorescence at either different time points or in different cell populations, and comparing the determinations to one another or to standards. In general, changes of at least about 50% from baseline are preferred, with changes of at least about 75%-100% being more preferred, changes of at least about 250% being particularly preferred, and changes of at least about 1000-2000% being especially preferred. Baseline in this case means the styryl dye uptake of cells prior to exocytic stimulation.
- Preferred styryl dyes include, but are not limited to FM1-43, FM4-64, FM14-68, FM2-10, FM4-84, FM1-84, FM14-27, FM14-29, FM3-25, FM3-14, FM5-55, RH414, FM6-55, FM10-75, FM1-81, FM9-49, FM4-95, FM4-59, FM9-40, and combinations thereof. Preferred dyes such as FM1-43 are only weakly fluorescent in water but very fluorescent when associated with a membrane, such that dye uptake is readily discernable. Suitable dyes are available commercially, i.e., Molecular Probes, Inc., of Eugene, Oregon, "Handbook of Fluorescent Probes and Research Chemicals", 6th Edition, 1996, particularly, Chapter 17, and more particularly, Section 2 of Chapter 17, (including referenced related chapter), hereby incorporated herein by reference. Preferably, the dyes are provided in a solution wherein the dye concentration is about 25 to 1000- 5000 nM, with from about 50 to about 1000 nM being preferred, and from about 50 to 250 being particularly preferred. The use of styryl dyes is further described in Betz, et al., Current Opinion in Neurobiology, 6:365-371 (1996) also incorporated herein by reference. Preferably, fluorescent dye uptake is measured in combination with at least one, and preferably two other indicators of exocytosis activity.
- In another preferred embodiment, changes in fluorescent dye release are evaluated. The present invention is in part directed to the discovery that low pH concentration dyes, which are normally used to stain lysosomes, also low pH stain exocytic granules. Generally, these dyes can be taken up by the cells passively and concentrate in granules; however, the cells can be induced to take up the dye, i.e., by coupled endocytosis. In a preferred embodiment, a cell population is bathed in a low pH concentration dye such that the dye is taken up by the cells. The cells are preferably washed. The cells can be exposed to a pro-exocytic stimulus and/or inhibitor. In a preferred embodiment, a candidate bioactive agent is combined with the cell population and preferably, the pro-exocytic stimulus. Fluorescence is evaluated. Changes in fluorescent dye release between cells or at different time points in the same cell indicate alterations in exocytosis. Preferably, the alterations are between cells, and most preferably, between cells having different bioactive agents added thereto. Changes of at least about 5% from baseline are preferred, with at least about 25% being more preferred, at least about 50% being particularly preferred and at least about 100% being especially preferred. Baseline in

this case means the amount of dye in the cells prior to stimulation.

In this embodiment, low pH concentration dyes are preferred. Such low pH concentration dyes include but are not limited to acridine orange, LYSOTRACKER™ red, LYSOTRACKER™ green, and  
5 LYSOTRACKER™ blue. Such dyes are commercially available, i.e., from Molecular Probes, supra, particularly including Chapter 17, Section 4 of Chapter 17, and referenced "related chapters", i.e., Chapter 23. In preferred embodiments, the dyes are administered in a solution wherein the dye is a concentration of about 50 nM to about 25  $\mu$ M, with from about 5  $\mu$ M to about 25  $\mu$ M being preferred, and from about 1 to 5  $\mu$ M being particularly preferred. The use of low pH concentration dyes is  
10 generally described (in regards to lysosome studies) in Haller, et al., Cell Calcium, 19(2):157-165 (1996), hereby incorporated herein by reference.

In an alternative embodiment wherein changes in fluorescent dye release are evaluated, the fluorescence released into the supernatant is evaluated. In this embodiment, either styryl dyes, which  
15 reversibly label endocytosed membranes, or low pH concentration dyes are used. In this embodiment, a cell population is bathed in dye such that the dye is taken up into the cells passively or by induction. The cells are then preferably washed. The cells can be exposed to a pro-exocytic stimulus and/or inhibitor, and optionally, a candidate bioactive agent. The cells which are exposed to a pro-exocytic stimulus will release the dye into the extracellular medium. The fluorescence in the medium can be  
20 measured or detected. This process is sometimes referred to as destaining the cells. Optionally, an agent for improving and facilitating the detection of the dye in the medium can be added. For example, micelle-forming detergents such as 3-[(3-cholamidopropyl)dimethylammonio] -1-propanesulfonate (CHAPS) increase the fluorescence and thereby allow detection of small amounts of exocytosis activity. Changes in the release of dye will indicate alterations in exocytosis in the same  
25 cell, between cells, and most preferably, between cells having different bioactive agents added thereto. In general, changes of at least about 5% from baseline are preferred, with at least about 25% being more preferred, with at least about 50% being particularly preferred and at least about 100% being especially preferred. Baseline in this case means the release of dye prior to exocytotic stimulus. Preferably, dye release when measured in the media is combined with the evaluation of at least one  
30 other exocytosis indicator.

In a preferred embodiment, changes in granule exposure are determined. The granules are exposed to the media during exocytosis, i.e., the granules fuse with the cell membrane and expose/release their contents. Therefore, granule exposure is indicative of exocytic activity, and its absence is  
35 indicative that exocytosis has not been induced, or has been inhibited. Preferably, granule exposure is detected by a detectable agent which specifically bind to granules. An example of a detectable agent used herein is annexin V, a member of a protein family which displays specific binding to

phospholipid (phosphatidylserine) in a divalent ion dependent manner. This protein has been widely used for the measurement of apoptosis (programmed cell death) as cell surface exposure of phosphatidylserine is a hallmark early signal of this process. Surprisingly, it has been determined herein that annexin V specifically binds to exocytic granules when they are exposed at the cell surface during the secretory process; granules internal to the cell are unlabeled. This property of annexin V is used herein to create a single exocytosis assay based on its exocytosis dependent binding. Upon exocytic stimulation of cells, the cells show an increase in annexin binding and fluorescent signal in proportion in both time and intensity to the exocytic response.

- 10 In this embodiment, annexin is labelled, either directly or indirectly, and combined with a cell population. Annexin is commercially available, i.e., from PharMingen, San Diego, California, or Caltag Laboratories, Millbrae, California. Preferably, the annexin is provided in a solution wherein the annexin is in a concentration of about 100 ng/ml to about 500 ng/ml, more preferably, about 500 ng/ml to about 1 µg/ml, and most preferably, from about 1 µg/ml to about 5 µg/ml. In a preferred  
15 embodiment, the annexin is directly labelled; for example, annexin may be labelled with a fluorochrome such as fluorescein isothiocyanate (FITC), Alexa dyes, TRITC, AMCA, APC, tri-color, Cy-5, and others known in the art or commercially available. In an alternate preferred embodiment, the annexin is labelled with a first label, such as a hapten such as biotin, and a secondary fluorescent label is used, such as fluorescent streptavidin. Other first and second labelling pairs can be used as  
20 will be appreciated by those in the art.

- In the preferred embodiment, the cells are subjected to conditions that normally cause exocytosis. Optionally, a candidate bioactive agent is added to the cells. In some cases, it may be desirable to include an inhibitor of exocytosis to determine whether the candidate agent can reverse the inhibition,  
25 or to add the candidate bioactive agent without an exocytic stimulus to determine whether the agent induces exocytosis. The cells are preferably washed and fluorescence is detected in the microscope or on the flowcytometer. Alterations in the detection of annexin binding indicates alterations in exocytosis in the same cell, or between different cells, with or with the same conditions and/or agents combined therewith. In general, changes of at least about 25% from baseline are preferred, with at  
30 least about 50% being more preferred, at least about 100 being particularly preferred and at least about 500% being especially preferred. Baseline in this case means the amount of annexin binding prior to exocytic stimulation.

- In another preferred embodiment, granule exposure is detected by a cationic dye such as berberine or  
35 ruthenium red. Such cationic dyes specifically stain secreting granules. Thus, when exocytosis occurs, and secreting granules are exposed at the cell surface, an increase in fluorescence can be detected. In a preferred embodiment, the cationic dye is combined with a cell population in the



presence or absence of an exocytic stimulus and/or inhibitor, and optionally, in the presence or absence of a candidate bioactive agent. In a particularly preferred embodiment, the berberine is combined with a cell and an exocytic stimulus and a candidate bioactive agent to determine whether the candidate bioactive agent can modulate the exocytic activity. Preferably, the cells are washed and  
5 then fluorescence is determined. In preferred embodiments, cationic dye evaluation is combined with evaluation of at least one other indicator of exocytosis. The dye is combined with the cells as is known in the art. General methodologies describing berberine are described in Berlin and Enerback, Int. Arch. Allergy Appl. Immunol., 73(3):256-262 (1984) hereby incorporated by reference. In general, changes of at least about 5% from baseline are preferred, with at least about 25% being more preferred, at  
10 least about 50% being particularly preferred, and at least about 100% being especially preferred. Baseline in this case means the amount of dye binding prior to stimulation.

Similarly, Con A-FITC can be used, as it binds to the carbohydrate on granule proteins, in a manner similar to those outlined herein.

15 In another preferred embodiment, changes in surface granule enzyme activity is determined. Secretory granules contain enzymes such as proteases and glycosidases which are released as part of the exocytic process. Frequently, these enzymes are inactive within the granule, due to the low pH, but upon exposure to the extracellular media at physiological pH, they become activated. These  
20 enzyme activities can be measured using chromogenic or fluorogenic substrates as components of the extracellular media. This allows detection of exocytic cells in varying approaches.

In one embodiment, sometimes called herein the population based enzyme assay, the generation of signal via cleavage of a chromogenic or fluorogenic substrate can be quantified in the media. That is,  
25 the amount of detectable reaction product in the media is related to the amount of enzyme present, and thus to the amount of exocytosis. In this embodiment, it is the media, not the cells, that becomes detectable.

In a preferred embodiment, cells are subjected to an exocytic stimulus, and optionally, a candidate  
30 bioactive agent. The chromogenic or fluorogenic substrate is added to the media, and changes in the signal are evaluated, as the enzymes cleave the extracellular substrates.

In an alternate preferred embodiment, sometimes called herein "in situ enzymology assay", fluorogenic substrates that precipitate upon cleavage are used. That is, upon exocytosis a considerable amount  
35 of enzyme activity remains cell/granule associated and can be visualized using fluorescent substrates which precipitate at the site of activity. For example, substrates for glucuronidase, such as ELF-97 glucuronide, precipitate on exocytosing cells, but not resting cells, and thus the cells can show

increased fluorescence. The fluorescence is a direct measurement of exocytosis and is pH dependent reflecting the pH optima of the exocytosed enzyme. This method also provides a method of distinguishing different subtypes of granules based on their enzyme profile.

- 5 In a preferred embodiment, the cell population is subjected to an exocytic stimulus and then incubated with a detectable substrate. A candidate bioactive agent is optionally added. The cells are washed and then viewed in the microscope or flowcytometer.

Preferred granule enzymes include but are not limited to chymase, tryptase, arylsulfatase A, beta-  
10 hexosaminidase, beta-glucuronidase, and beta-D-galactosidase. Substrates include ELF-97 glucuronide, N-acetyl beta-D glucuronide, ELF-97 coupled to peptides, etc., many of which are commercially available, i.e., from Molecular Probes, supra, particular Chapter 10, more particularly Section 2 of Chapter 10, and referenced "related chapters".

- 15 By detectable substrate is meant that the substrate comprises a fluorescent molecule as further described herein, or can be detected with a fluorescent molecule specific for the substrate or cleaved substrate, i.e., a fluorescent antibody. In a preferred embodiment, the substrate comprises a detectable molecule formed of two fluorescent proteins, i.e., blue and green fluorescent protein (BFP and GFP), and other similar molecules. As is known in the art, constructs of GFP and BFP that hold  
20 these two proteins in close proximity allow fluorescence resonance energy transfer (FRET). That is, the excitation spectra of the GFP overlaps the emission spectra of the BFP. Accordingly, exciting the BFP results in GFP emission. If a protease cleavage site is engineered between the GFP and BFP to form a "FRET construct", upon exposure of the FRET construct to an active protease which cleaves the construct, the GFP and BFP molecules separate. Thus, exciting the GFP results in BFP emission  
25 and loss of BFP emission.

Preferably, the protease dependent cleavage site inserted between two fluorescent proteins of the FRET construct is specific for a granule specific enzyme. Thus, the FRET construct can be used for detecting granule specific proteases specific for the cleavage site of the FRET construct. In this  
30 embodiment, the protease substrate that is combined with the cells or media includes the FRET construct. The FRET system allows for detection of the detectable molecule in its cleaved and uncleaved state, and distinguishes between the two. The system is further described in Xu et al., Nucleic Acid Res. 26(8):2034 (1998); and Miyawaki et al., Nature 388(6645):882-887 (1997), both of which are incorporated by reference.

35

The amount of substrate added to the cells or media will depend in part on the enzyme's specific activity and the substrate itself, but generally is about 250 nM to about 1 mM, from about 1  $\mu$ M to

about 100  $\mu$ M being preferred, and from about 1  $\mu$ M to about 10  $\mu$ M being particularly preferred. In general, changes of at least about 5% from baseline are preferred, with at least about 25% being preferred, at least about 100% being particularly preferred and at least about 1000% being especially preferred. Baseline in this case means the amount of substrate cleavage prior to induction of  
5 exocytosis.

In a preferred embodiment, changes in the quantity of granule specific proteins are determined. Secretory granules contain proteins which are specifically targeted to the granule compartment due to specific properties of these proteins. Upon exocytic induction, the granule specific proteins are  
10 exposed to the surface and detected.

In a preferred embodiment, detectable granule specific proteins are combined with a population of cells and subjected to conditions known to induce exocytosis. Optionally, a bioactive candidate is combined with the cell population and detectable granule specific protein and the granule specific  
15 protein is detected. Granule specific proteins include but are not limited to VAMP and synaptotagmin. Also included within the definition of granule specific proteins are the mediators released during exocytosis, including, but not limited to, serotonin, histamine, heparin, hormones, etc.

The quantification of the granule proteins may be done in several ways. In one embodiment, labelled  
20 antibodies, (such as fluorescent antibodies), to granule specific proteins are used. In another embodiment, the cells are engineered to contain fusion proteins comprising a granule protein and a detectable molecule. In a preferred embodiment, a detectable molecule is added to the cells for detection. For example, either directly or indirectly labelled antibodies can be used. A preferred  
25 embodiment uses a first labelled antibody, with fluorescent labels preferred. Another embodiment uses a first and second label, for example, a labelled secondary antibody. Generally, this embodiment may use any agent that will specifically bind to the granule protein or compound that can be either directly or indirectly labelled.

In a preferred embodiment the labels are engineered into the cells. For example, recombinant  
30 proteins are introduced to the cell population which are fusion proteins of a granule specific protein and a detectable molecule. This is generally done by transforming the cells with a fusion nucleic acid encoding a fusion protein comprising a granule specific protein and a detectable molecule. This is generally done as is known in the art, and will depend on the cell type. Generally, for mammalian cells, retroviral vectors and methods are preferred.

35 The fusion proteins are constructed by methods known in the art. For example, the nucleic acids encoding the granule specific protein is ligated with a nucleic acid encoding a detectable molecule. By

- detectable molecule herein is meant a molecule that allows a cell or compound comprising the detectable molecule to be distinguished from one that does not contain it, i.e., an epitope, sometimes called an antigen TAG, or a fluorescent molecule. Preferred fluorescent molecules include but are not limited to GFP, BFP, YFP, enzymes including luciferase and  $\beta$ -galactosidase. These constructs can be
- 5 made in such a way so that upon exocytosis an epitope, internal to the granule, is exposed at the cell surface and can then be detected. The epitope is preferably any detectable peptide which is not generally found on the cytoplasmic membrane, although in some instances, if the epitope is one normally found on the cells, increases may be detected, although this is generally not preferred.
- 10 In a preferred embodiment, the cell population containing the fusion protein or detectable granule specific protein is subjected to exocytic conditions. Optionally, a candidate bioactive agent and/or exocytic inhibitor is included. Preferably, the cells are washed. Fluorescence is detected on the cells. In general, changes of at least about 5% from baseline are preferred, with at least about 25% being more preferred, at least about 50% being particularly preferred and at least about 100% being
- 15 especially preferred. Generally, baseline in this case means amount of fluorescence prior to exocytic stimulus.

In the invention herein, the same characteristic of exocytosis is evaluated by multiple parameters which results in reduced background and greater specificity. In contrast, FACS has been used in the

20 past to evaluate two different or unrelated characteristics at the same time which identifies cells having those two characteristics, but does not reduce the background for the combined characteristics. The present invention can, however, in addition to the identification of multiple exocytosis properties, be combined with the identification of other cellular parameters, as outlined above.

- 25 In a preferred embodiment, the cells are subjected to conditions that normally cause exocytosis. Pro-exocytic agents include ionomycin,  $\text{Ca}^{++}$ , ionophores (Ionomycin, AZ3187), compound 48/80, substance P, complement C3a/C5a, trypsin, tryptase, insulin, interleukin-3, specific IgE, allergen, anti-IgE, or anti-IgG receptor antibodies. These are provided at concentrations depending on the compound as is known in the art, ranging from 1 picomolar to 10  $\mu\text{M}$ , generally. In some cases, it
- 30 may be desirable to combine the cells with agents which inhibit exocytosis. Exocytosis inhibitors include but are not limited to Wortmannin, and Genestein, and others known in the art.

- In a preferred embodiment, the methods are used to screen candidate bioactive agents for the ability to modulate exocytosis. The candidate bioactive agents may be combined with the cell population
- 35 before, during or after exocytosis is stimulated, preferably before. In some instances, it may be desirable to determine the effect of the candidate bioactive agent, also referred to as "candidate agents" herein, on the cell wherein exocytosis is not induced or wherein exocytosis is inhibited. The

candidate bioactive agent can be added to the cell population exogenously or can be introduced into the cells as described further herein.

5 In a preferred embodiment, as above for cell cycle assays, a library of different candidate bioactive agents are used.

As above, the candidate bioactive agents are combined or added to a cell or population of cells; again, as outlined above, preferred embodiments utilize nucleic acid candidate agents and fusion partners; and preferably retroviral constructs.

10 Wherein the candidate agents are nucleic acids, methods known in the art such as calcium phosphate, electroporation, and injection may be used to introduce these to the cells. The exocytic stimulus is generally combined with the cells under physiological conditions. Incubations may be performed at any temperature which facilitates optimal activity, typically between 4 and 40°C. Incubation periods  
15 are selected for optimum activity, but may also be optimized to facilitate rapid high through put screening.

As above, a variety of other reagents may be included in the assays, and the cells are sorted as above. The sorting results in a population of cells having the desired exocytic properties. In a  
20 preferred embodiment, the parameters are set to identify at least one candidate bioactive agent that modulates exocytosis.

In a preferred embodiment, the bioactive agent is characterized. This will proceed as will be appreciated by those in the art, and generally includes an analysis of the structure, identity, binding  
25 affinity and function of the agent. Generally, once identified, the bioactive agent is resynthesized and combined with the target cell to verify the exocytosis modulation under various conditions and in the presence or absence of other various agents. The bioactive can be prepared in a therapeutically effective amount to modulate exocytosis and combined with a suitable pharmaceutical carrier.

30 In a preferred embodiment, the cell populations can be subjected to various experimental conditions, with and without the candidate agents, and with and without exocytic stimulation or inhibition. Changes in conditions include but are not limited to changes in pH, temperature, buffer or salt concentration, etc. In a preferred embodiment, the pH is changed, generally by increasing or decreasing the pH, usually by from about 0.5 to about 3 pH units. Alternatively, the temperature is  
35 altered, with increases or decreases of from about 5°C to about 30 °C being preferred. Similarly, the salt concentration may be modified, with increases or decreases of from about 0.1 M to about 2 M being preferred.

In a preferred embodiment, the cellular phenotype to be modulated is small molecule (or other candidate agent) toxicity. These are generally as outlined above for cell viability assays. Small molecule dose responses can also be compared by comparing the cells with the greatest functional response, and then backgating to see if there is more or less toxicity associated with those cells.

5

In a preferred embodiment, the cellular phenotype involves the expression or activity of cell surface receptors; up to sixteen cell surface markers may be followed simultaneously, with up to eight being preferred. The presence or absence of any particular cell surface marker can be detected by directly and indirectly conjugated antibodies against any cell surface protein whose cell surface expression reflects an important functional parameter associated with the cells being studied. The effect of candidate agents such as small molecules can then be tested against individual or multiple markers.

10

In a preferred embodiment, the cellular phenotype involves the expression or activity of enzymes such as fluorescent based reporter systems that can report a biological event that occurs simultaneously with the primary measurement or is a result of the primary measurement. This reporter system can be a readout of upstream signal transduction pathways that are active in the cytoplasm, or of nuclear transcriptional or translational events, as well as export events from the nucleus or the cell.

15

In a preferred embodiment, the cellular phenotype involves protein-protein interactions (or interactions between other binding ligands), such as dimerization, that can be either disrupted or instigated by a candidate agent. These events may be measured by the appearance or disappearance of FRET between two labeled binding ligands.

20

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are expressly incorporated by reference in their entirety.

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30

#### EXAMPLE 1

##### Cell Cycle Assays using p21 as a positive control

##### Materials and Methods:

Vector Construction: The coding region of the p21 gene was cloned from Jurkat cDNA by PCR with an upstream primer covering the start methionine (5'-GATCGGATCCACC ACCATGGGCTCAGAACCGGCTGGGGATGTC) and C-terminus (5'-GATCCC AATTTAATGGTTTTATTTGTCATCGTCATCCTTGTAGTCGGGCTTCCTCTTGGAGAAGATCAGCCG

35

- GCGTTTG). The single PCR product was directionally cloned into the CRU5-GFP retroviral vector (Rigel, Inc.) through flanking BstXI sites within the primers. The resultant construct, CRU5-GFP-p21F (Figure 1), encodes the GFP fused (in frame) to the human p21 protein with a Gly insertion at position 2 and a FLAG-epitope at the C-terminus. The C-terminal 24 amino acids of p21 were cloned into the
- 5 CRU5-GFP retroviral vector (Rigel, Inc.) through flanking BstXI sites within the PCR primers: 5' GATCCCACCACCATGGGCAAACGGCGGCAGACCAGCATGACAGATTCTACCACTCCAAACGCC GGCTGATCTTCTCAA;
- 5'GATCCCAATTTAAATGGTTTTATTTGTCATCGTCATCCTTGTAGTCGGGCTTCCTCTTGGAGAAG ATCAGCCGGCGTTTG. The resultant construct, CRU5-GFPp21C (Figure 1), encodes GFP fused in-
- 10 frame to KRRQTSMTDFYHSRRLIFSKRKP and a FLAG-epitope at the C-terminus. The C-terminal 24 amino acids of p21, with three alanine mutations, were cloned into the CRU5-GFP retroviral vector (Rigel, Inc.) through flanking BstXI sites within the PCT primers:
- 5'ATCGGATCCACCACCATGGGCAAACGGCGGCAGACCAGCGCCACAGCTGCCTACCACTCC;
- 5'GATCCCAATTTAATGGTTTTATTTGTCATCGTCATCCTTGTAGTCGGGCTTCCTCTTGGAGAAGA
- 15 TCAGCCGGCGTTTG. The resultant construct, CRU5-GFPp21Cmut (Figure 1), encodes GFP fused in-frame to KRRQTSATAAYHSRRLIFSKRKP (mutations are underlined) and a FLAG-epitope at the C-terminus.

- Retroviral Transduction: Phoenix E cells were plated in 6-well plates at  $10^6$  cells in 1.5 ml complete-
- 20 DMEM (DMEM + 10% FBS + Pen/Strep) and incubated at 37°C for 16 hours.  $\text{CaCl}_2$ -precipitation transfection was performed (2  $\mu\text{l}$  DNA (1  $\mu\text{g}/\mu\text{l}$ ), 30.5  $\mu\text{l}$  2M  $\text{CaCl}_2$ , 217.5  $\mu\text{l}$   $\text{H}_2\text{O}$ , 0.5 ml 2X HBS) with the CRU5-IRES-GFP vector or CRU5-p21F-IRES-GFP clone in the presence of 50  $\mu\text{M}$  chloroquine for 8 hours at 37°C. The transfection-medium was removed and replaced with 2 ml complete-DMEM and the cells were further incubated for 16 hours at 37°C. The medium was changed to 1.5 ml complete-
- 25 RPMI (RPMI + 10% FBS + Pen/Strep) and incubated at 32°C for 48 hours. The virus supernatant from transfected plates was filtered (0.45 $\mu\text{m}$ ) and transferred to a 6-well plate. An 100  $\mu\text{l}$  aliquot ( $5 \times 10^6$  cells) of Jurkat T-cells expressing the ecotrophic receptor (JurkatE) was added to each well. Polybrene was added to a final concentration of 5  $\mu\text{g}/\text{ml}$ . The plates were sealed with parafilm and centrifuged at 32°C for 90 minutes at 2500 RPM. The parafilm was removed and the plate incubated
- 30 overnight at 37°C. The medium was changed after 16 hours to 4 ml complete-RPMI and incubated at 37°C for 72 hours.

- Cell Cycle FACS-Assay: The retroviral vector-transduced cells were pelleted and resuspended at  $10^6$  cells/ml in complete-RPMI. One volume (1ml) of 4  $\mu\text{M}$  PKH26 cell tracking dye (Sigma) was added to
- 35 the cells and incubated at 25°C for 5 minutes. The suspension was diluted 5-fold and the cells pelleted at 400 x g for 10 minutes at 25°C. The cells were further washed twice with 6 ml complete-RPMI and incubated at  $3 \times 10^5$  cells/ml in a 6-well plate for 72 hours. The labeled cells were pelleted

and resuspended at  $10^6$  cells/ml in complete-RPMI containing 5 ug/ml Hoechst 33342 (Molecular Probes) and incubated at 37°C for 2 hours. The stained cells were pelleted and resuspended at  $>10^6$  cells/ml in FACS buffer (PBS/0.5%FCS/5ug/ml Hoechst 33342). The cells were subjected to flow-cytometric analysis on a MoFlo cytometer (Cytomation) equipped with three lasers. Forward and side scatter were triggered with a 488nm-line argon laser and scattered light was collected with a forward scatter detector and 488nm band pass filter. GFP was excited with a 488nm-line argon laser and emitted light was collected through a 530nm-band pass filter. PKH26 cell tracking dye was excited with a 533nm-line HeNe-laser and emitted light was collected through a 570nm-band pass filter. Hoechst 33342 dye was excited with a UV-laser and emitted light was collected through a 450nm-band pass filter.

#### Results:

Jurkat T-cells were transduced with with retroviral vectors encoding human p21 (Gp21), or the PCNA binding C-terminal 24 amino acids (Gp21C) fused to GFP (Figure 1). A non-PCNA binding mutant version of the p21 C-terminal 24 amino acids (Gp21Cmut, Cayrol et al., Oncogene 16:311 (1998)) served as a negative control. Expression of the transduced p21 could be distinguished from the endogenous protein by the FLAG-epitope by Western blotting (not shown). Expression of the fusion proteins was reported in the FACS by GFP fluorescence (Figure 2B). Transduced cells were pulsed labeled with a cell tracking compound, pkh26, which incorporates red fluorescent aliphatic molecules into the cell membrane by selective partitioning, allowing a correlation between cell cycling and fluorescent intensity: arrested cells remain cell tracker dye bright; cycling cells dilute the signal and dim. As shown in Figure 2C, live GFP-p21-expressing cells gated on GFP, demonstrated a higher red fluorescence than vector transduced cells expressing identical GFP levels, indicating cell cycle arrests. A similar effect was seen in the Gp21C expressing cells, however, Gp21-Cmut was identical to non-expressing cells. The DNA content of the same GFP-gated cells is shown in Figure 2D. Gp21 expressing cells are arrested in the G1 phase of the cell cycle, Gp21C-expressing cells show G1 and G2 checkpoint accumulation, consistent with previous results (Wade Harper, et al., 1993; Cayrol et al., 1998). The Gp21Cmut expressing cells show a normal cell cycle distribution. Viable, arrested, expressing cells (satisfying the three initial parameters) were sorted based on DNA content into separate chambers: left deflection, G1; right deflection, G2.

## EXAMPLE 2

### Population Based Exocytic Enzyme Activity Measurements

**Materials:** All chemicals were obtained from Sigma Chemical Co. Dyes and glucuronide were obtained from Molecular Probes, Inc. Cell lines MC-9 and RBL-2H3 were obtained from American Type Culture Collection (ATCC). Cell culture reagents were obtained from Fisher Scientific and



molecular biology reagents from Clontech Inc.

Cell Culture: MC-9 cells were maintained as suspension cultures in flasks in media consisting of DMEM with L-arginine (116 mg/ml), L-asparagine (36 mg/ml), sodium pyruvate (1 mM), non-essential amino acids (0.1 mM), folic acid (6 mg/ml), 2-mercaptoethanol (0.05 mM), L-glutamine (2 mM), heat inactivated fetal bovine serum (10%), and 10% T-stim conditioned media (Collaborative Research, Inc.). The cells were kept at a density of between .25 and  $2 \times 10^6$  /ml. Experiments were only conducted on cells which were greater than 95% viable as determined by trypan blue exclusion. RBL-2H3 cells were maintained as adherent cultures on uncoated (tissue culture treated) flasks in media consisting of Eagles MEM with 2mM L-Glutamine and Earl's BSS, 15% heat inactivated fetal bovine serum. The cells were passaged (.05% trypsin) so that they were not confluent for more than one day.

Exocytosis Stimulation Protocol: Experiments were carried out in modified tyrodes buffer (MT) which consisted of NaCl (137 mM), KCl (2.7 mM),  $\text{CaCl}_2$  (1.8 mM),  $\text{MgCl}_2$  (1 mM), Glucose (5.6 mM), Hepes (20 mM, pH 7.4), and bovine serum albumin (0.1%). MC-9 cells were spun at  $400 \times g$  and the media was aspirated. The cells were then washed with MT, respun/aspirated and taken up in MT at a density of  $5 \times 10^6$  cells /ml. Cells were then treated with either DMSO or ionophore for 30 minutes (or the time was varied if a timecourse). The cells were then pelleted with the supernatant collected for enzymatic analysis; in some cases, the cells then processed for flow cytometry. All stimulations were carried out at  $37^\circ\text{C}$ . Stimulations of RBL-2H3 cells were carried out by washing the adherent cells one time in MT and then adding warmed MT (1 ml/ $10^6$  cells) containing the stimulus. The cells were incubated at  $37^\circ\text{C}$  for 30 minutes and the supernatant was harvested for further analysis. In some of the examples, (Examples 4-6), the plate bound cells were stained for annexin and then removed from the flask using No-Zyme (Collaborative Research, Inc.) for further processing for flow cytometry. For stimulation of RBL-2H3 cells with antigen crosslinking the cells were incubated overnight with IgE anti-DNP (Sigma Chemical Co.) in complete media at a concentration of 50 ng/ml. The following day they were washed one time in MT and stimulated as described above with the exception that bovine serum albumin coupled to DNP was used as the stimulus at 100 ng/ml.

Population Based Enzyme Assays: Enzyme assays were carried out on cell supernatants and pellets following exocytic stimulation. Cell supernatants were harvested after stimulation, chilled on ice, and the post  $5000 \times g$  spin supernatant was collected for enzyme activity analysis. Similarly, cell pellets were collected/lysed in MT containing 0.1% triton X-100 and the post  $5000 \times g$  spin supernatant was collected for enzyme activity analysis. For each analysis 100  $\mu\text{l}$  of lysate or supernatant was mixed with 100  $\mu\text{l}$  of reaction buffer (40mM Citrate, pH 4.5) containing 2mM substrate (4-methylumbelliferyl  $\beta$ -D Glucuronide [glucuronidase substrate] or 4-methylumbelliferyl N-acetyl  $\beta$ -D glucosaminide

(hexosaminidase substrate)) in a solid black 96 well plate (Costar, Inc.) and incubated at 37°C for 15 minutes. The plate was read on a fluorescence plate reader (Wallac, Inc.) using excitation 380 nm/emission 440 nm filters every 3 minutes for five times to obtain an enzymatic rate; analyses were carried out in triplicate.

5

**Flow Cytometry:** Cells processed for stimulation and staining were taken up in MT on ice and filtered through a 100 µm filter prior to cytometry. Cells were analyzed using a FACSCAN (Becton Dickinson Inc., laser line 458 nm) or a Mo-Flo (Cytomation, Inc., laser lines 350nm broadband (UV), 488nm, and 647nm) Cytometer. Cells were sorted, if desired using the Mo-Flo.

10

**Results:** The results are shown in Figure 4. Enzymatic activity in the cell supernatant was measured for MC9 (A) and RBL 2H3 (B) cells under various conditions. A) MC-9 cells were stimulated in the presence of DMSO (-) or 2 µm Ionomycin (+) for 30 minutes. The supernatant was collected and analyzed for glucuronidase or hexosaminidase activity. Stimulated release of granule enzymatic activity is evident. B) RBL-2H3 cells were sensitized for 16 hours with varying amounts of IgE anti-DNP and stimulated to exocytose by exposure to increasing amounts of the antigen BSA-DNP. A dose response of both antibody and antigen is evident in the measured supernatant hexosaminidase activity.

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20

### **EXAMPLE 3: Mast Cell Exocytic Light Scatter Changes**

The cells were prepared as described in Example 2, and light scatter properties were determined.

**Results:** The results are shown in Figure 4. Light scatter changes observed on the flow cytometer (side scatter vs. forward scatter) are plotted as bivariate histograms for RBL-2H3 cells (A, D) and MC-9 cells (B, C, E, F). Cells were stimulated with the ionophore A23187 (0.5 µg/ml) and observed at various timepoints [0 minutes (A, C), 5 minutes (E), 10 minutes (D), and 30 minutes (B, F)]. Time dependent scatter changes are evident in both cell lines with significant changes occurring during the first 10 minutes which represents the major bolus of exocytosis in these cells.

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### **EXAMPLE 4: Styryl Dyes Detect Mast Cell Exocytosis by FACS**

**Styryl Dye Staining:** The cells were prepared as described above. Styryl dyes (FM1-43 or FM4-64; Molecular Probes, Inc.) were diluted to a final concentration of 250 nM in MT and were incorporated into the stimulation buffer (see Example 2). After the stimulation protocol the cells were spun down, aspirated and resuspended in fresh ice cold MT. The cells were then ready for analysis in the flow cytometer (see Example 2).

35

**Results:** The results are shown in Figure 6. MC-9 cells were stimulated (blue = DMSO, red = 2  $\mu$ M ionomycin) in the presence either FM 4-64 (A, B) or FM 1-43 (C,D,E). A) FM 4-64 labeled cells detected in the flow cytometer in fluorescence channel 1. B) FM 4-64 labeled cells detected in the flow cytometer in fluorescence channel 3. C) FM 1-43 labeled cells detected in the flow cytometer in fluorescence channel 1. D) FM 1-43 labeled cells detected in the flow cytometer in fluorescence channel 3. There is a clear stimulation dependent increase of fluorescence intensity with both dyes; FM 4-64 being the most red-shifted and predominantly detected in channel 3 while FM 1-43 is more broadly fluorescent being detected in both channels 1 and 3. E) MC-9 cells were preincubated with varying doses of the PI-3 kinase inhibitor wortmannin (1  $\mu$ M-bar1, 100 nM-bar2, 10 nM-bar3, and 0 nM-bars 1 and 2) prior to stimulation with A23187 (0.5ug/ml, bars 1-4) or DMSO (bar 5) in the presence of FM 1-43. The mean channel shift detected in the flow cytometer in fluorescence channel 1 is plotted as a bar graph. Wortmannin, a known inhibitor of mast cell exocytosis, causes a dose dependent decrease in the FM 1-43 signal indicating that FM 1-43 signal reflects the degree of degranulation in the MC-9 mast cell line.

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#### **EXAMPLE 5: Annexin-V Staining Detects Mast Cell Exocytosis by FACS**

**Materials:** Annexin-V biotin, Annexin-V FITC and streptavidin APC were obtained from Caltag Laboratories. Other materials and methods used herein can be incorporated from the other examples, particularly Example 2.

20

**Annexin-V Staining:** Cells post exocytic stimulus were stained with annexin-FITC at a dilution of 1/100 in MT for 10 minutes at room temperature. The cells were then washed one time in MT, taken up in MT and viewed in the flow cytometer or microscope. For indirect labeling, annexin-biotin was added to the MT during the stimulation procedure at a dilution of 1/200. The cells were then pelleted in ice cold MT, spun, aspirated, and taken up in ice cold MT with streptavidin-APC at a dilution of 1/200 and kept on ice for 15 minutes. After pelleting the cells and aspirating the Streptavidin-APC, the cells were resuspended in MT and viewed in the flow cytometer. In some experiments different secondaries were applied such as streptavidin alexa 488 or 594 (Molecular Probes, Inc.) for visualization in the microscope.

30

**Results:** The results are shown in Figure 7. MC-9 cells were stimulated with either DMSO (Figures A and B) or 2  $\mu$ M ionomycin (Figures C and D) and then stained with both propidium iodide [PI] (Figures A and C) and annexin-V-FITC (Figures B and D). Stimulation with this dose of ionomycin does not compromise the plasma membrane as demonstrated by no significant increase in PI staining in the exocytosing cells. Degranulation results in a significant increase in annexin binding as seen comparing Figures D and B.

35

**EXAMPLE 6: Annexin-V-FITC stains exocytic granules in MC-9 cells visualized by confocal microscopy**

- 5 **Microscopy:** Cells post stimulation or staining were mounted onto glass slides and coverslipped; these were directly visualized by brightfield and fluorescence microscopy on an inverted microscope (TE300, Nikon) using standard BFP, FITC, or TRITC filter sets. Some images were obtained using an inverted confocal scanning microscope (Zeiss, Inc., Bio-Rad, Inc.) using standard FITC and TRITC filter sets.
- 10 **Results:** MC-9 cells were stimulated with 2  $\mu$ m ionomycin or DMSO and stained with annexin-V-FITC and mounted for confocal microscopy (data not shown). All viable unstimulated cells show low annexin binding and no cell surface granular staining.

**EXAMPLE 7: Annexin-V-FITC Stains Exocytic Granules In RBL-2H3 Cells Stimulated with Antigen Crosslinking**

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Except as otherwise stated below, the methods for this example are described in the preceding examples.

- 20 **Results:** RBL-2H3 cells were sensitized with IgE and stimulated to exocytosis with either MT buffer only or BSA-DNP antigen (100 ng/ml) and then stained with annexin-V-FITC. Numerous cell surface granules are stained in the antigen stimulated cells similar to the pattern seen in MC 9-cells. Viable unstimulated cells show negligible annexin-V staining (data not shown).

25 **EXAMPLE 8: In Situ Enzymology of Exocytosing Cells Visualized in the FACS**

- In Situ Enzymology:** MC-9 cells were stimulated for exocytosis as described above and then incubated in enzyme substrate buffer (BSA free MT, pH 4.3 - 7.4 range) containing the substrate ELF-97 Glucuronide (250  $\mu$ M) for 15 minutes at 37°C. The cells were washed one time in MT and
- 30 then viewed in the microscope or on the flowcytometer. Further methodologies are described in the preceding examples.

- Results:** The results are shown in Figure 8. MC-9 cells were stimulated with DMSO (Figure A) or A23187 (0.5  $\mu$ g/ml - Figure B and C) and then stained for in situ glucuronidase activity. A) Flow
- 35 cytometer histogram of ELF-97 detection indicative of cell surface enzymatic precipitate. Very low signal is seen in the DMSO treated cells. B) Flow cytometer histogram of ELF-97 detection indicative of cell surface enzymatic precipitate. A significant increase in signal is seen upon secretory

stimulation with ionophore. C) pH profile of the cell surface enzymatic activity. MT buffer, prepared at different pHs, was used to pH profile the signal seen in the flow cytometer. The bar graphs represent the percentage of maximal signal (as measured by mean channel shift in the flow cytometer) observed. The enzymatic activity is pH dependent with a peak at less than pH 6; this is consistent with  
5 enzymatic activity derived from an acidic secretory granule.

**EXAMPLE 9: Lysotracker Green Is Released from Mast Cell Granules upon Exocytosis and Can Be Detected by FACS**

- 10 **Lysotracker Dye Staining:** Lysotracker dyes (blue, green, and red) were loaded into cells by diluting them to a final concentration of 1  $\mu$ M in complete media and incubating the cells for 60 minutes at 37°C in their presence. After loading, the cells were washed two times in MT and then were ready for further analysis or stimulation. Further methodologies are described in the preceding examples.
- 15 **Results:** The results are shown in Figure 9. MC-9 cells were loaded with Lysotracker green for 1 hour and then stimulated with either DMSO or ionomycin (2  $\mu$ M) and viewed in the flow cytometer. Shown is a histogram of fluorescence intensity detected in channel 1; a significant loss of signal is seen in the ionophore stimulated sample as compared to the DMSO control which is reflective of the release of lysotracker green dye from the secretory granules.

20

**EXAMPLE 10: Multiparameter Analysis--Lysotracker Green, Annexin-V-APC, Forward and Side Scatter**

Except as otherwise described below, the methodologies described in the preceding examples were  
25 used.

- Results:** The results are shown in Figure 10. MC-9 cells were treated with different doses of ionomycin (0  $\mu$ M- A, E; 1  $\mu$ M- B, F; 2  $\mu$ M- C, G; and 3  $\mu$ M- D, H) and observed in the flow cytometer with four parameters simultaneously. The cells were loaded with lysotracker green for one hour and  
30 then stimulated and stained for annexin-V-APC. Figures A-D: Bivariate histograms of side vs. forward light scatter. Note the dose dependent changes in both parameters from left to right as forward scatter increases and side scatter decreases. Figures E-H: Bivariate histograms of annexin-V-APC vs. Lysotracker green signals. As exocytosis increases (left to right) annexin signal becomes greater as the lysotracker signal decreases. This reflects the binding of annexin-V to the cell surface granules  
35 and the loss of lysotracker from these granules as they are exposed to the extracellular milieu.

**EXAMPLE 11: Multiparameter Analysis--FM 1-43, Annexin-V-APC, Forward and Side Scatter**

Except as otherwise noted below, the methodologies described above were used herein.

**Results:** MC-9 cells were treated with either DMSO or ionomycin (2  $\mu$ M) and observed in the flow cytometer with four parameters simultaneously. The cells were stimulated in the presence of FM 1-43 and stained for annexin-V-APC (data not shown). There were stimulation dependent changes in both parameters at the 30 minute timepoint. There were stimulation dependent increases in both signals. The changes reflect the binding of annexin-V to the cell surface granules and the simultaneous coupled endocytosis of the FM 1-43 dye into the MC 9 cells.

**EXAMPLE 12: Simultaneous Multiparameter Measurements in the FACS Correlate with Population Based Enzyme Readouts**

**Calcium Signaling Assays:** MC-9 or RBL-2H3 cells were washed one time in MT and loaded with the  $\text{Ca}^{++}$  sensitive probe Fluo-3 (1  $\mu$ M, Molecular Probes, Inc.) in MT at 37°C for 20 minutes. The cells were washed one time in warm MT and then stimulated using the protocol described above. The signal due to rise in the intracellular  $\text{Ca}^{++}$  concentration was visualized using either the flow cytometer (see below), fluorescence microscopy, or read on a fluorescence plate reader (Wallac, Inc.). Loading of the cells was determined by releasing the intracellular dye with MT containing 0.1 % triton X-100.

Except as otherwise noted below, the methodologies described above were used herein.

**Results:** The results are shown in Figure 11. MC-9 cells were stimulated in the presence of FM 1-43 and annexin-V-APC stained as described in the methods above. At various timepoints after ionomycin stimulation the cells were put on ice and either analyzed by flow cytometry or for enzymatic activity (cell supernatant). The parameters forward scatter, FM 1-43, annexin-V-APC, and hexosaminidase are plotted on the graph relative to the maximal response for each parameter. For calcium signaling, a separate tube of cells was loaded with Fluo-3 and underwent the identical procedure. The timecourses of the cytometry based parameters indicate that they correlate quite well with exocytosis as measured by hexosaminidase release. Forward scatter, in this example, shows an effect which varies both positively and negatively with time.

**EXAMPLE 12: Expression of VAMP-GFP and VAMP-FRET Constructs**

**cDNA Constructs:**

**VAMP-GFP construct:** The rat VAMP-2 cDNA (obtained from R. Scheller, Stanford University) was PCR modified to introduce: (1) a 5' BstXI site encoding a consensus Kozak and glycine insertion

- (a.a.2) to facilitate expression and *in vivo* stability, respectively; (2) a serine-glycine linker with a BamHI site at the 3' end. The GFP coding sequence from CdimGFP (Clontech, Inc.) was PCR modified to introduce a 3' BstXI site encoding a stop codon. The VAMP-GFP fusion was constructed by ligating the modified rVAMP and GFP PCR fragments through a common BamHI site in the
- 5 serine-glycine linker to create an in-frame fusion protein with the following sequence:

MGSATAATVPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLSEDD  
RADALQAGASQFETSAAKLKRKYWWKNLKMMIILGVICAILIIIVYFST  
 GSGSGSGSGSGSPVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTTKFICTTGKLPV  
 10 PWPTLVTTLTHGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVN  
RIELKGIDFKEDGNILGHKLEYNFNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGD  
GPVLLPDNHYLSTQSALSKDPNEKRDHMLLEFVTAAGITLGMDELYKZ

- The VAMP sequence is underlined, the serine-glycine linker is italicized and the GFP sequence is in
- 15 regular text.

- The VAMP-GFP fusion sequence was cloned into the 96.7 retroviral vector with directional BstXI sites to create pVG. The sequence was verified by sequencing in both directions. Proper expression was verified in transfected and infected cells by Western analysis and fluorescence microscopy.

20

- Trp-FRET construct:** The GFP coding sequence from cGFP (Clontech, Inc.) was PCR modified to create: (1) a 5' BstXI site encoding a consensus Kozak and glycine insertion (a.a.2) to facilitate expression and *in vivo* stability, respectively; (2) a 3'-end SacII site encoding Ala228 at the C-terminus. The BFP coding sequence from cBFP (Clontech, Inc.) was PCR modified to create: (1) a 5' BamHI
- 25 site encoding Ser2; (2) a 3'-end BstXI encoding a stop codon. A SacII-BamHI conversion linker encoding Factor X and tryptase protease cleavage sites, flanked by GSGS spacers (GSGSIEGRLRKQGSCS) was used to fuse the GFP and BFP to create an in-frame fusion protein with the following sequence:

30 MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTTKFICTTGKLPVPWPTLVTTLTYG  
VQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDG  
NILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLST  
QSALSKDPNEKRDHMLLEFVTAAGSGSIEGRLRKQGSGSKGEELFTGVVPILVELDGDVNGHKFSV  
SGEGEGDATYGKLTTKFICTTGKLPVPWPTLVTTLTHGVQCFSRYPDHMKQHDFFKSAMPEGYVQER  
 35 TIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYIMADKQKNGIKVNF  
KIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMLLEFVTAAGITLGMDELYKZ

The GFP sequence is underlined, the Factor X/tryptase site linker is italicized and the BEP sequence is in regular text.

5 The VAMP-GFP fusion sequence was cloned into the BamHI and BstXI sites of the retroviral vector 96.7 to create pGX/TB. The sequence was verified by sequencing in both directions. Proper expression was verified in transfected and infected cells by Western analysis and fluorescence microscopy.

10 The VAMP-GFP encoding sequence was PCR modified to create a 3'-end SacII site encoding Ala228 at the C-terminus. This fragment was cleaved with XhoI and SacII and cloned into the XhoI/SacII sites of pGX/TB to create pVGX/TB (Trp-FRET), encoding the rVAMP-2-BFP-FactorX/Tryptase sites-GFP fusion protein with the following sequence:

GSATAATVPPAAPAGEGGPPAPPPNLTSNRRLLQQTQAQVDEVVDIMRVNVDKVLERDQKLSELD  
DDR  
ADALQAGASQFETSAAKLKRKYWWKNLKMMLILGVICAILIIIVVFSTGSGSGSGSGSGSPV  
 15 SKGEELFTGVWPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKICTTGKLPVPWPTLVTTLTTHGVQ  
 CFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNIL  
 GHKLEYNFNHSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYSTQS  
 ALSKDPNEKRDHMLLEFVTAAGSGSIEGRRKLQSGSGSKGEELFTGVWPILVELDGDVNGHKFSVSG  
 EGEEDATYGKLTCLKICTTGKLPVPWPTLVTTLTLYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIF  
 20 FKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNHSHNVYIMADKQKNGIKVNFKIR  
 HNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYSTQSALSKDPNEKRDHMLLEFVTAAGITLGMDEL  
 YKZ

25 The VAMP sequence is underlined, the serine-glycine linker is italicized, the Factor X/tryptase site linker is in bold and the GFP and BFP sequences are in shown in regular text.

The rVAMP-2-BFP-FactorX/Tryptase sites-GFP fusion sequence was verified by sequencing in both directions. Proper expression was verified in transfected and infected cells by Western and fluorescence microscopy and FACS analysis.

30

Transfections and Infections: To infect MC-9 and RBL-2H3 cells with recombinant retroviruses expressing the Vamp constructs the following procedure was carried out Phoenix E or A cells (obtained from G. Nolan, Stanford Univ.) were plated out in 6 well plates at  $8 \times 10^5$  cells in 1.5 ml media (DMEM, 10% FBS) on day one. On day two 5 µg of DNA was transfected into the cells using 35 the CaPO<sub>4</sub> precipitation method in the presence of 50 µM chloroquine. The precipitate was incubated with the cells for 8 hours at 37°C at which time the medium was removed, washed once with fresh media and replaced with either fresh MC-9 or RBL-2H3 media; the cells were then incubated at 32°C



- for 48-72 hours. The supernatant from the Phoenix cells (viral supernatant) was spun at 1000 xg for 10 minutes and protamine sulfate was added to a final concentration of 5 µg/ml; this supernatant was added to the MC-9 or RBL-2HS freshly trypsinized) cells in a 6 well plate (5 x 10E5 cells per well) and the mixture was spun at 1000 x g for 90 minutes at room temperature. The cells were then incubated at
- 5 32°C for 16 hours. The viral supernatant was removed and fresh media was added; target gene expression was seen after 24 hours post infection.

## CLAIMS

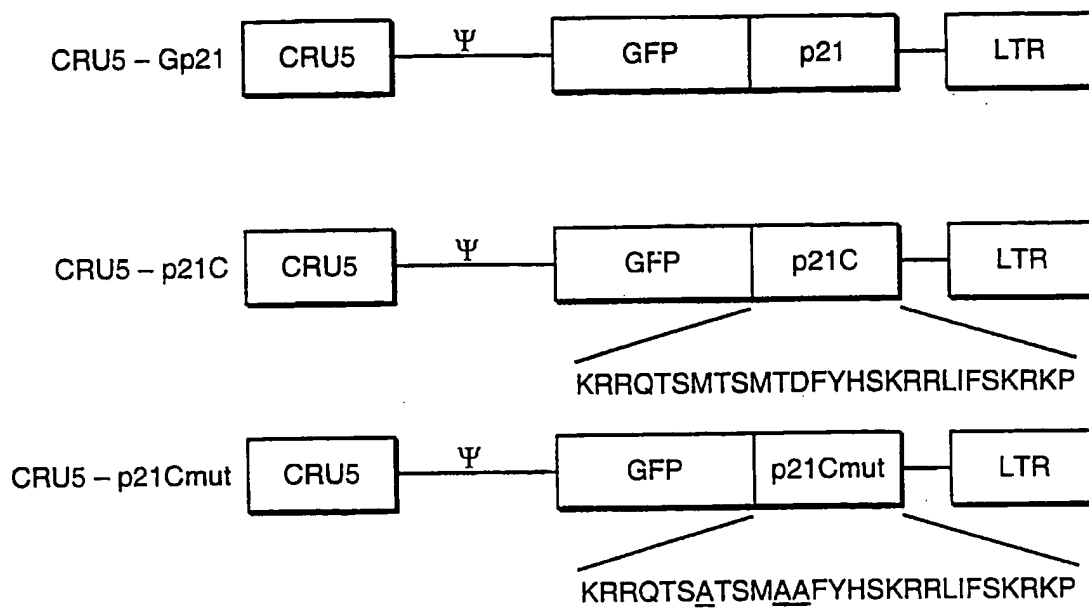
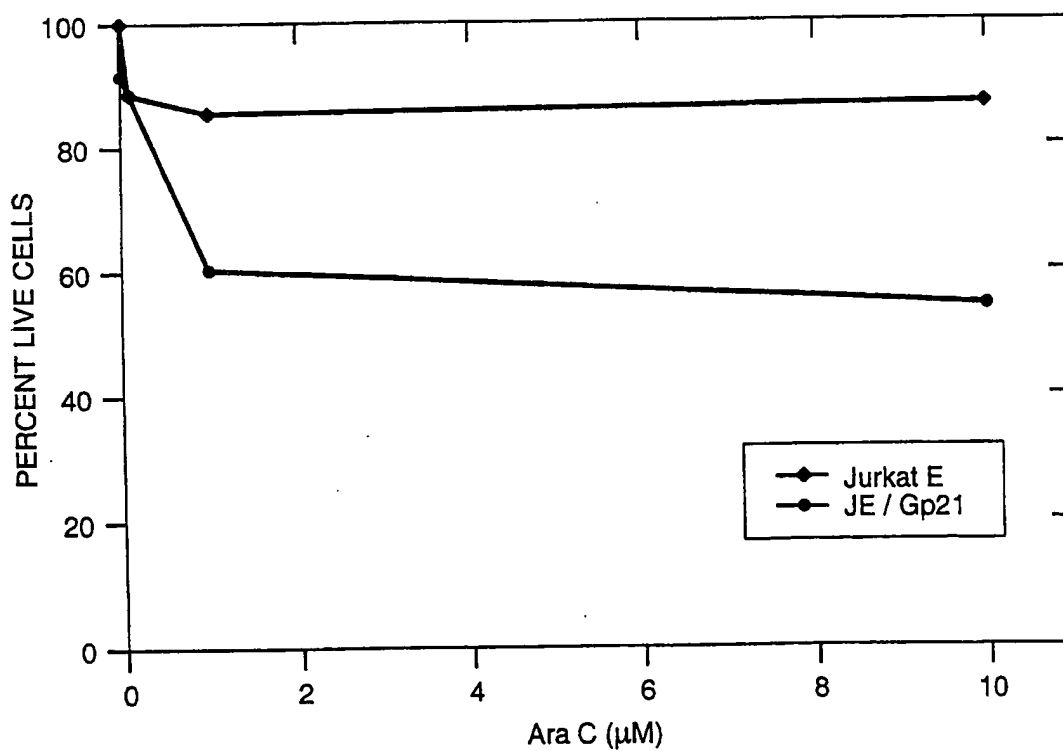
We claim:

1. A method of screening for a bioactive agent capable of altering a cellular phenotype, said method  
5 comprising:
  - a) combining at least one candidate bioactive agent and a population of cells; and
  - b) sorting said cells in a FACS machine by separating said cells on the basis of at least five cellular parameters.
- 10 2. A method according to claim 1 wherein a library of candidate bioactive agents are combined with said population.
3. A method of screening for a bioactive agent capable of altering a cellular phenotype, said method comprising:  
15
  - a) introducing a library of nucleic acids each encoding a candidate bioactive agent into a population of cells; and
  - b) sorting said cells in a FACS machine by separating said cells on the basis of at least three cellular parameters.
- 20 4. A method according to claim 3 wherein said library is a retroviral library.
5. A method according to claim 3 or 4 wherein said cellular phenotype is exocytosis and said cellular parameters are selected from the group consisting of light scattering, fluorescent dye uptake, fluorescent dye release, annexin granule binding, surface granule enzyme activity, and the quantity of  
25 granule specific proteins.
6. A method according to claim 5 further comprising subjecting said cells to conditions that normally cause exocytosis.
- 30 7. A method according to claim 3 or 4 wherein said cellular phenotype is cell cycle regulation and said cellular parameters comprise cell viability, proliferation, and cell phase.
8. A method according to claim 3, 4, 5, 6 or 7 wherein said nucleic acids comprise fusion nucleic acids comprising:  
35
  - a) said nucleic acid encoding said candidate bioactive agents; and
  - b) a detectable moiety.

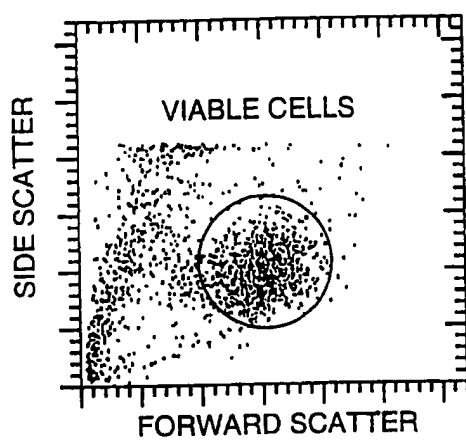
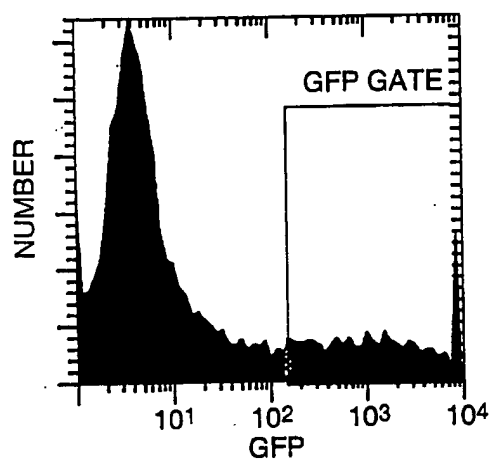
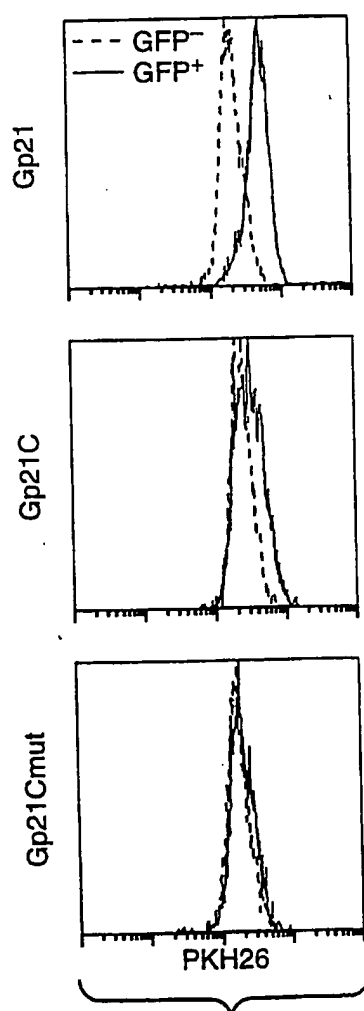
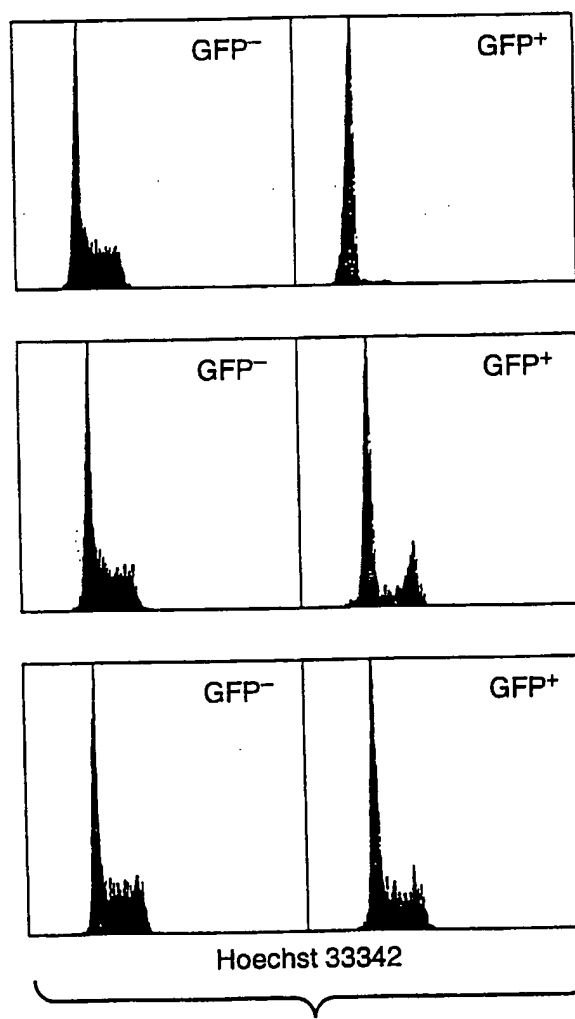
9. A method according to claim 1, 2, 3, 4, 5, 6, 7 or 8 wherein said cells are tumor cells.
10. A method according to claim 8 wherein said detectable moiety is a fluorescent protein.

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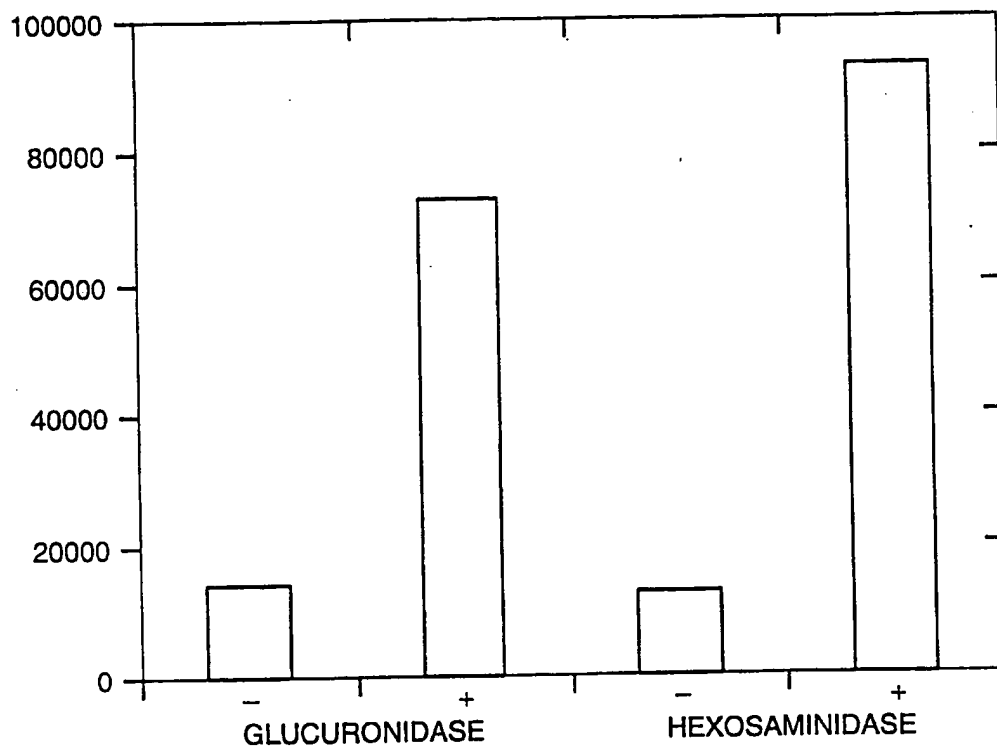
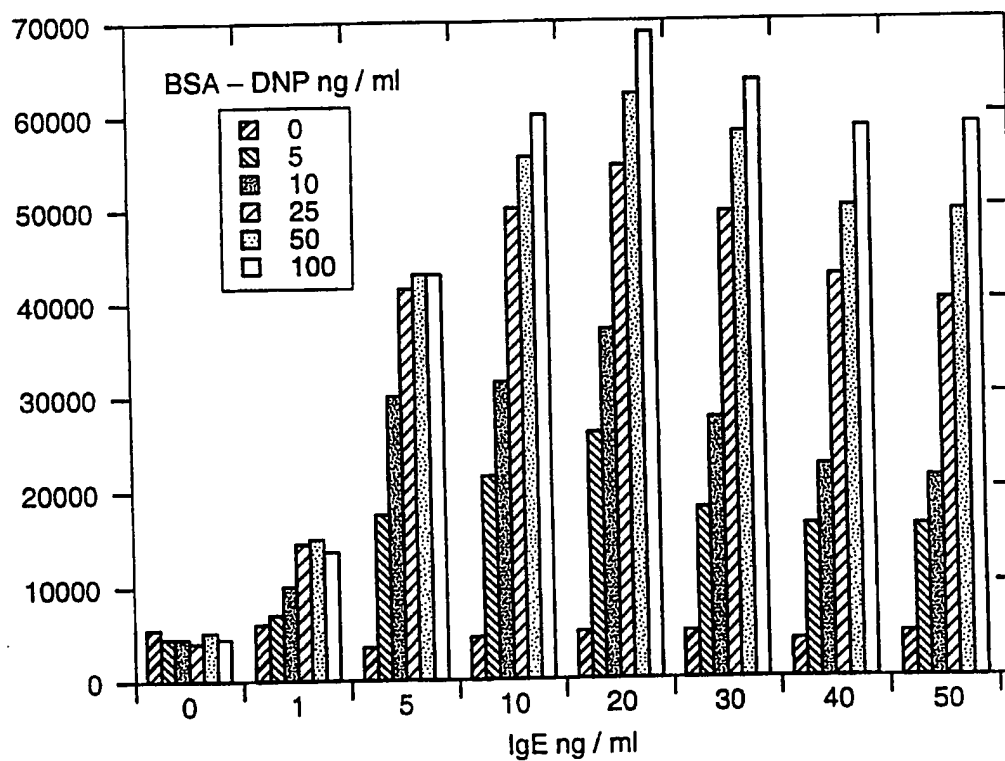
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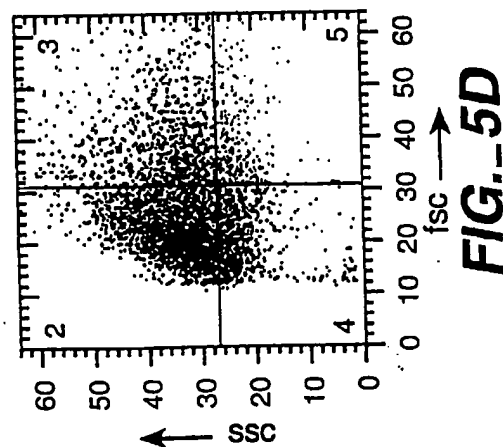
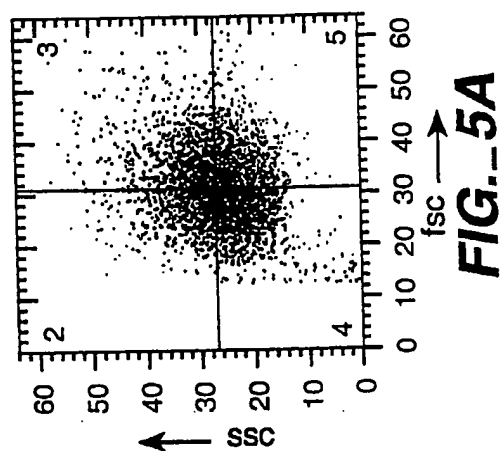
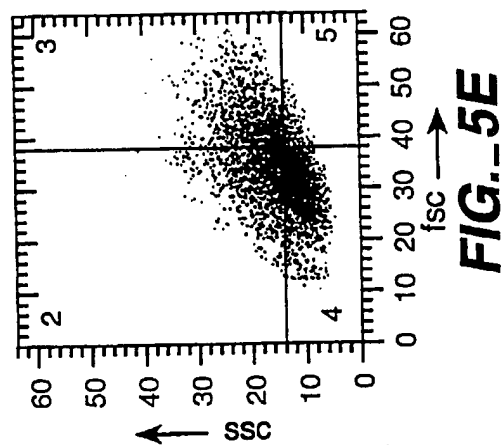
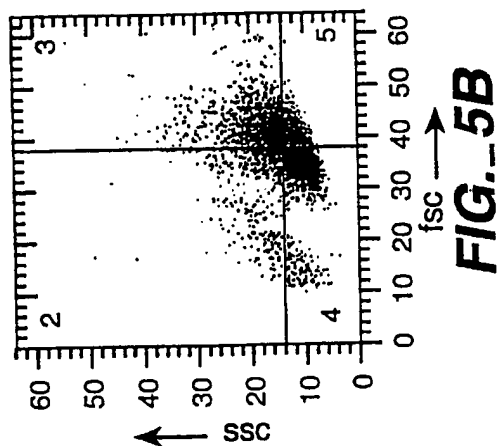
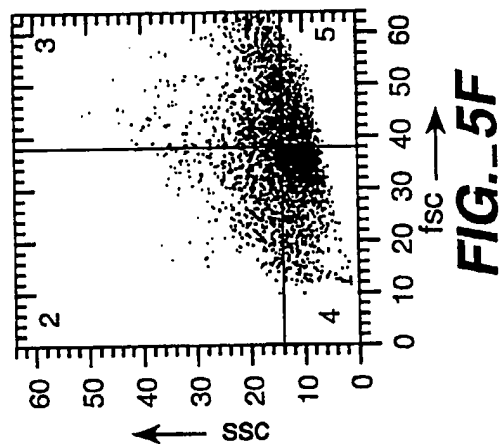
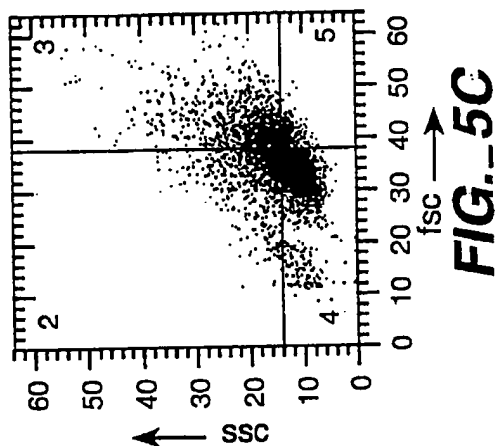
**FIG.\_1****FIG.\_3**

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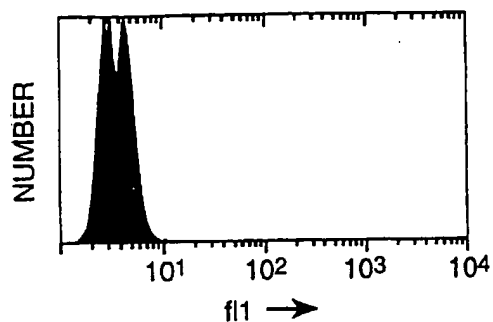
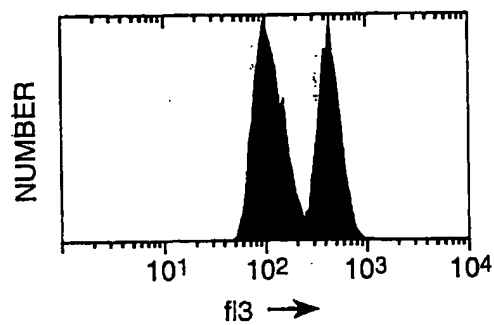
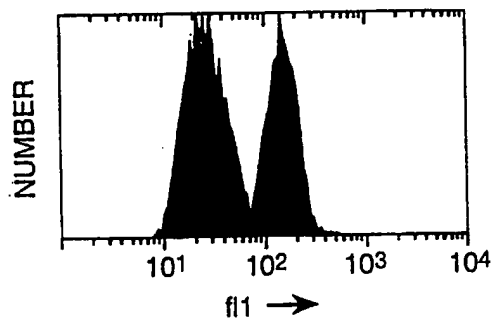
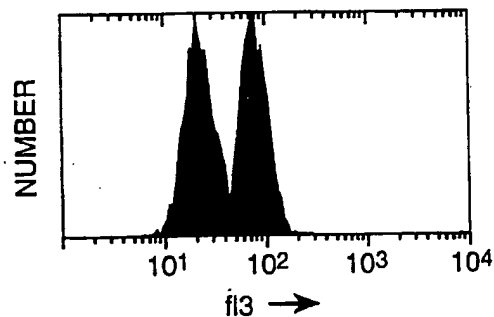
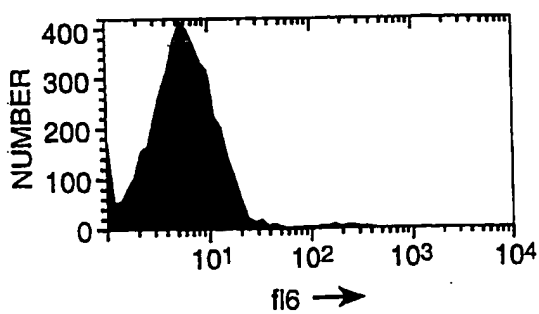
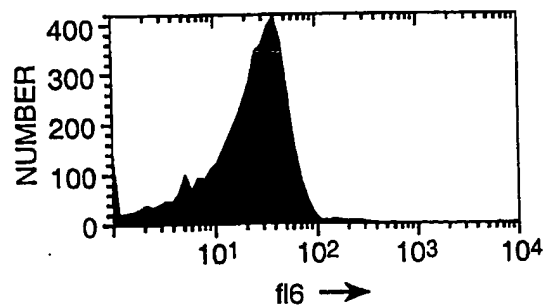
**FIG. 2A****FIG. 2B****FIG. 2C****FIG. 2D**

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**FIG. 4A****FIG. 4B**

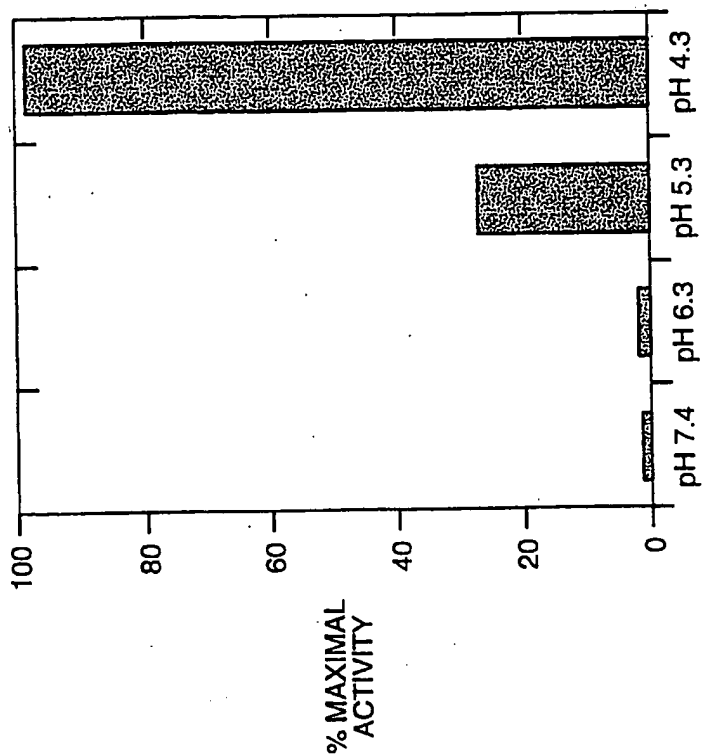


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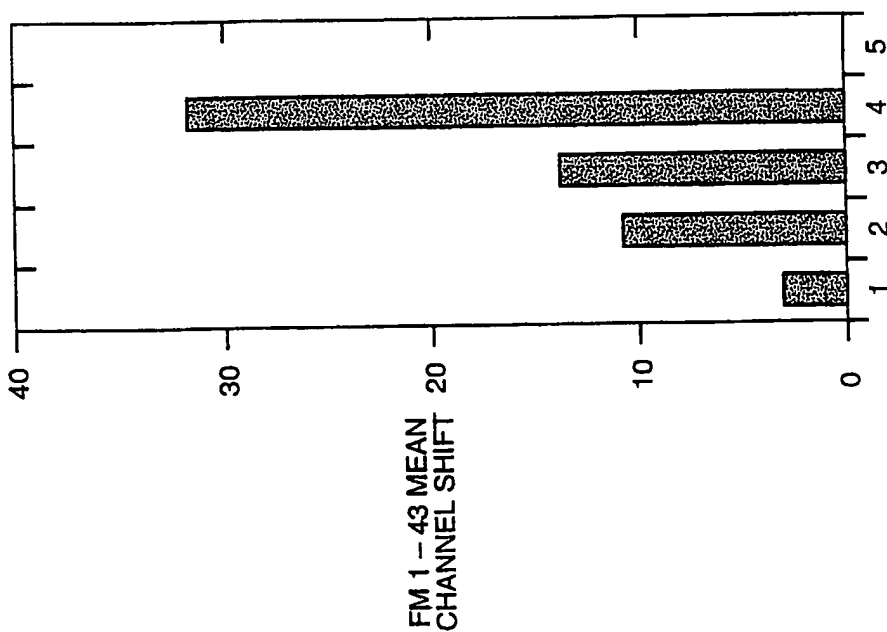
**FIG. 6A****FIG. 6B****FIG. 6C****FIG. 6D****FIG. 8A****FIG. 8B**



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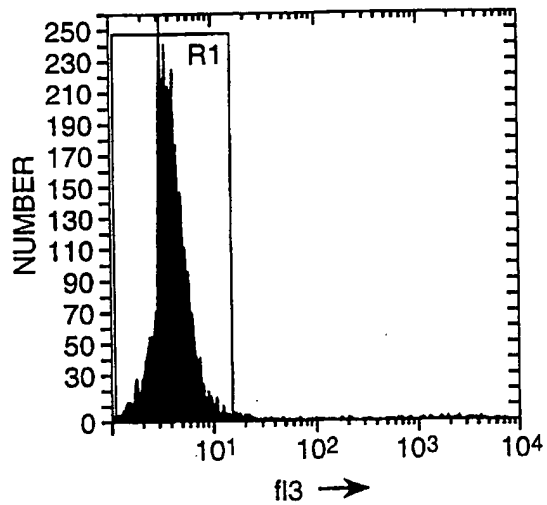


**FIG. 8C**

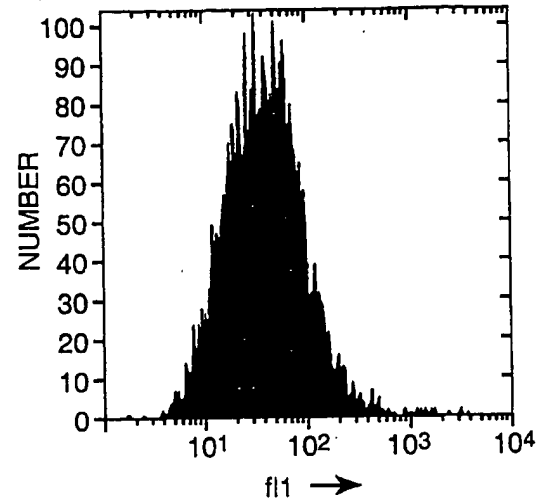


**FIG. 6E**

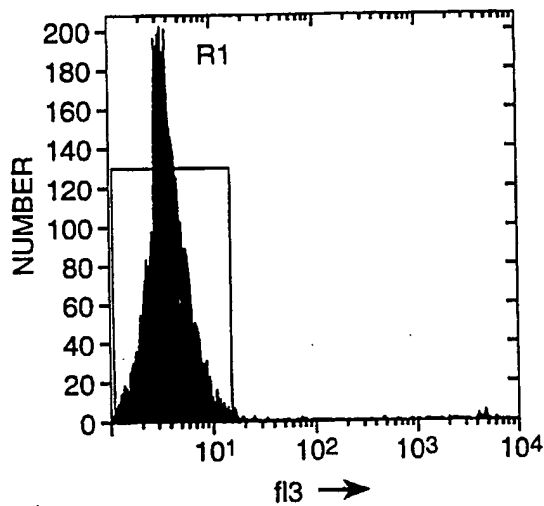
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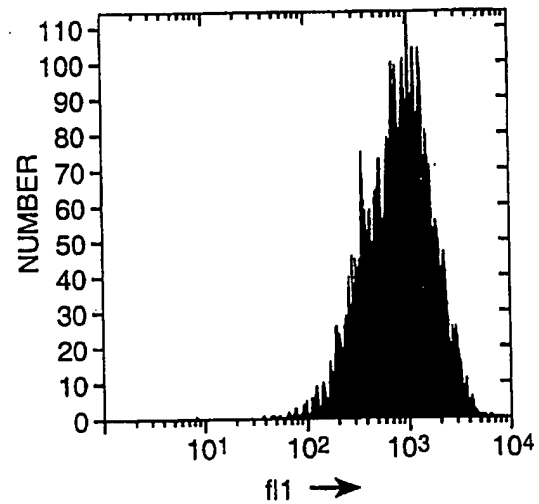
PROPIDIUM IODIDE

**FIG. 7A**

ANNEXIN - FITC

**FIG. 7B**

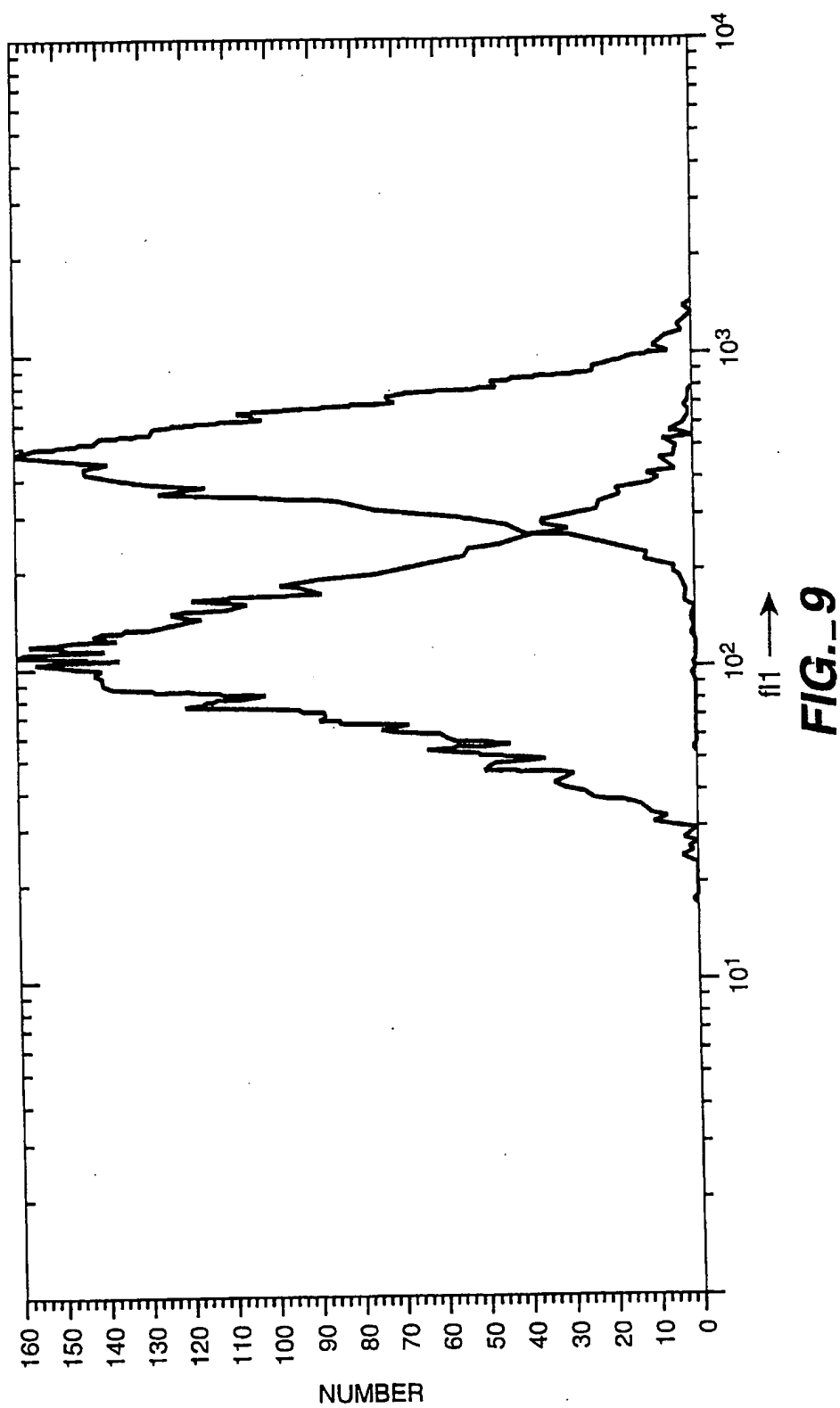
PROPIDIUM IODIDE

**FIG. 7C**

ANNEXIN - FITC

**FIG. 7D**

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**FIG. 9**

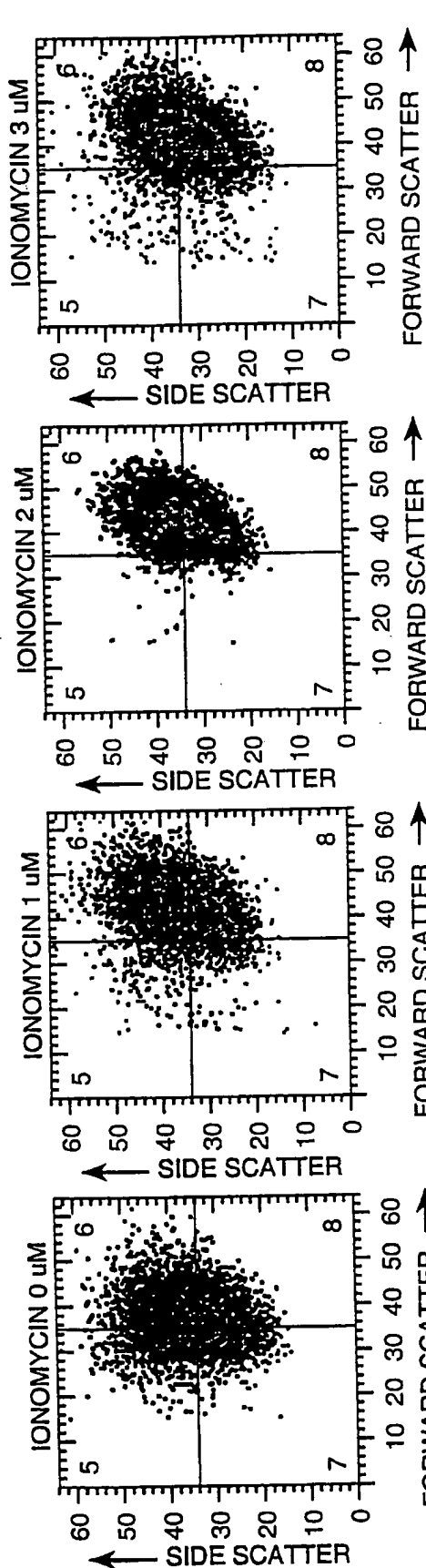


FIG. 10D

FIG. 10C

FIG. 10B

FIG. 10A

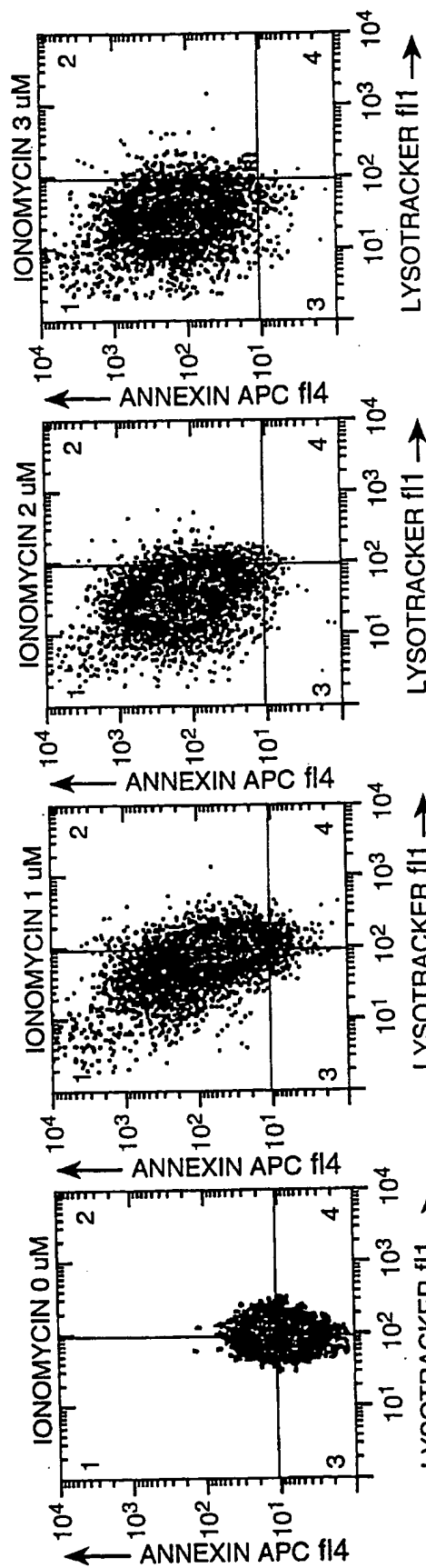


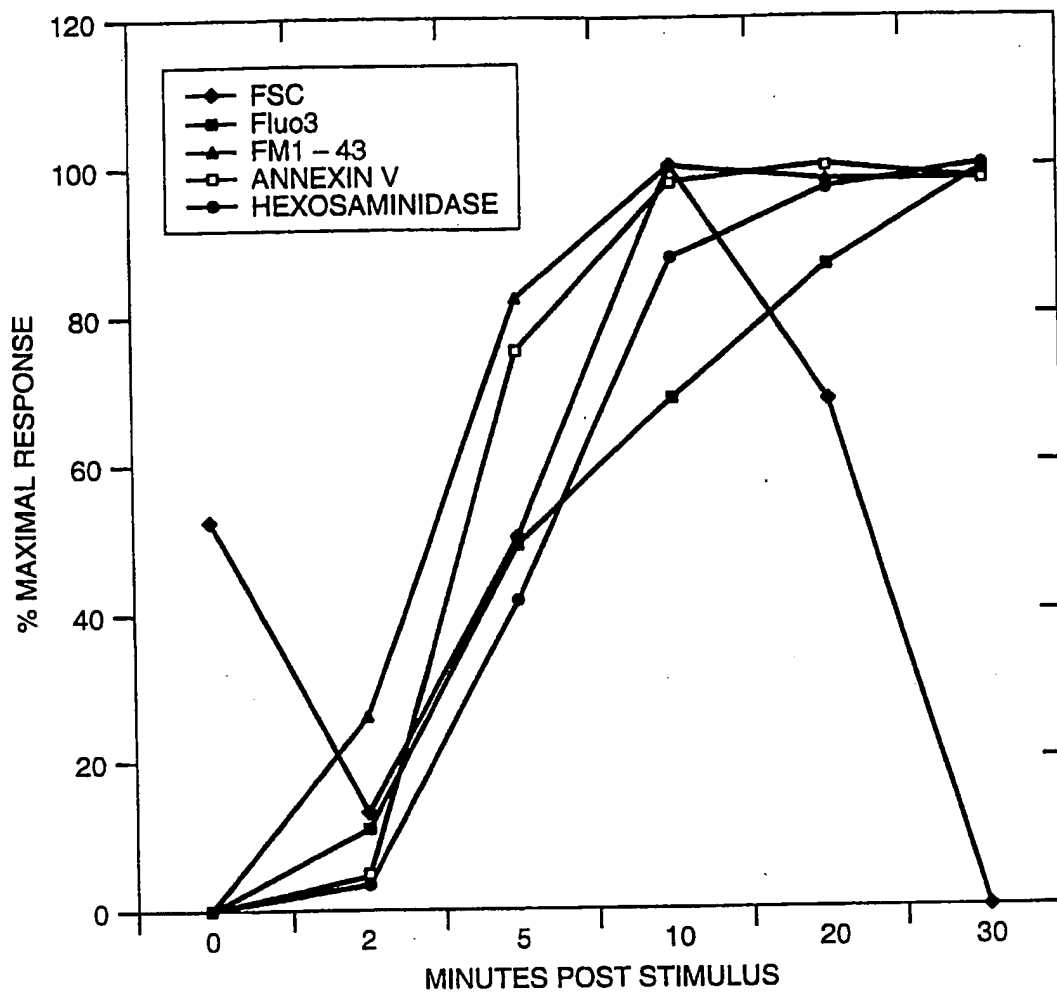
FIG. 10H

FIG. 10G

FIG. 10F

FIG. 10E

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**FIG.\_11**

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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			<b>(43) International Publication Date:</b> 28 October 1999 (28.10.99)
<b>(21) International Application Number:</b> PCT/US99/08345		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
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<b>(71) Applicant:</b> RIGEL PHARMACEUTICALS, INC. [US/US]; 240 E. Grand Avenue, South San Francisco, CA 94080 (US).			
<b>(72) Inventors:</b> FISHER, Joseph; 2634 Belmont Canyon Road, Belmont, CA 94002 (US). LORENS, James; 2010 Avy Avenue, Menlo Park, CA 94025 (US). PAYAN, Donald; 24 Windsor Drive, Hillsborough, CA 94010 (US). ROSSI, Alexander; 2177 - 48th Avenue, San Francisco, CA 94116 (US).			
<b>(74) Agents:</b> SILVA, Robin, M. et al.; Flehr, Hohbach, Test, Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).			
<b>(54) Title:</b> MULTIPARAMETER FACS ASSAYS TO DETECT ALTERATIONS IN CELLULAR PARAMETERS			
<b>(57) Abstract</b>  The invention relates to novel methods of detecting alterations in cell cycle regulation in a cell or a cell population and screening for agents capable of modulating cell cycle regulation through the use of multiparameter assays and a fluorescence-activated cell sorter (FACS) machine.			

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# INTERNATIONAL SEARCH REPORT

Internati Application No  
PCT/US 99/08345

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 G01N33/50 G01N33/533

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

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## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	BEUTH, J. ET AL: "Determination of lectin-dependent alterations of cellular parameters by immunophenotyping during adjuvant lectin application" LECTINS GLYCOBIOL. (1993), 396-401. EDITOR(S): GABIUS, HANS-JOACHIM; GABIUS, SIGRUN. PUBLISHER: SPRINGER, BERLIN, GERMANY. , XP000856690 the whole document	1
A	WO 91 02085 A (HOUSEY GERARD M) 21 February 1991 (1991-02-21) the whole document	1,3

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